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# Peroxisome Proliferator-Activated Receptor Delta (PPARD)

## Molecular studies of regulation and activation

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*To my family*

# ABSTRACT

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors involved in energy homeostasis. Their natural ligands are fatty acids and there are three different PPAR isoforms; PPARA, PPARG and PPARD. They are encoded by separate genes and have distinct functions, due to different tissue expression and affinity for ligands. PPARA controls genes involved in fatty acid oxidation, PPARG regulates genes important for fatty acid storage, and PPARD controls genes implicated in lipid oxidation and lipoprotein metabolism. Primates and humans treated with a PPARD agonist (GW501516) resulted in improved insulin sensitivity, increased HDL and decreased LDL cholesterol levels, making it a putative drug candidate for treatment of metabolic disease. PPARD has recently been assigned a beneficial role in macrophages, by inducing a switch from proinflammatory (M1) to antiinflammatory (M2) macrophages.

To characterize additional target genes of PPARD involved in the lipoprotein metabolism, the effect of PPARD activation on the apolipoprotein A-II (apoA-II) gene was investigated in human hepatoma cells. ApoA-II is one of the major proteins in the HDL particles. Treatment with GW501516 increased apoA-II promoter activity and mRNA levels in hepatoma cell lines. A site located at -737/-717 in the promoter was identified as the functional PPAR response element (PPRE). These results suggest that increased expression of the apoA-II gene is one of the reasons for the beneficial effects on lipoprotein metabolism after treatment with the PPARD agonist.

To investigate whether PPARs could regulate the alanine aminotransferase (ALT) genes, the effect of PPARA, G and D agonist treatment was studied. ALT activity in plasma is used as a marker for hepatotoxicity in humans. During a clinical trial with the PPARA ligand, AZD4619, the plasma ALT activity increased in some patients and *in vitro* studies showed that ALT1 protein and mRNA expression was induced by treatment with PPARA agonists in primary hepatocytes. Similarly, transient transfection of a promoter construct of ALT1 in HuH-7 cells showed increased activity mediated via a PPRE located at -574 after treatment with PPAR agonists. This study shows that the ALT1 gene is regulated by PPARs and that PPAR drugs might contribute to increased ALT activity in serum.

To explore regulation of the PPARD gene by posttranscriptional events, 5'- and 3'-RACE were performed on cDNA obtained from placenta, adipose tissue and pancreas. Both 5'- and 3'-alternative splicing of PPARD was identified. Coupled transcription/translation showed that the length and number of upstream AUGs in the 5'-UTR had a major impact on translational efficiency. Further, the promoter located upstream of exon one was verified as the major promoter, using reporter gene assays. A 3'-splice variant encoding a truncated PPARD protein, PPARD2, was shown to be a negative regulator of the full length receptor, PPARD1, in transient transfection assays.

To identify whether PPARD is regulated by microRNA (miRNA), the 3'-UTR was analysed *in silico*. Two putative miRNA target sites were identified in the PPARD 3'-UTR; miR-9 and miR-29. The miR-9 was verified as a functional miRNA targeting PPARD. However, PPARD mRNA levels remained unaffected by miR-9 expression, indicating that only the translation of PPARD was inhibited. Since both miR-9 and PPARD have been shown to play important roles in the inflammatory response of monocytes, the regulation of PPAR expression by miR-9 was investigated in these cells. A suppressive role of miR-9 on PPARD expression was identified in monocytes after LPS treatment but not in M1 or M2 macrophages, suggesting that the regulatory role of miR-9 on PPARD is exerted in monocytes, before differentiating into macrophages.

In summary, this thesis describes additional functions and ways of regulation of the ubiquitously expressed transcription factor PPARD with a major role in both health and disease.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt- och kärlsjukdomar såsom hjärtinfarkt, hjärtsvikt och stroke (slaganfall) är de vanligaste dödsorsakerna i västvärlden. Hjärt- och kärlsjukdomar beror av en fettansamling i kärlen under många decennier som med tiden leder till åderförkalkning, så kallad ateroskleros. När åderförkalkningen blockerar kärl drabbas vävnader av syrebrist vilket leder till sjukdom. Uppkomst av åderförkalkning beror av flera faktorer, där övervikt, höga blodfetter, högt blodtryck och högt blodsocker är de viktigaste. Dessa faktorer påverkas i sin tur av ärftlighet men även av våra livsvanor, där rökning, fysisk inaktivitet, samt matvanor spelar en huvudsaklig roll.

Peroxisom proliferator-aktiverade receptorer (PPAR) är en grupp proteiner som reglerar gener som håller kroppen i jämvikt vid ökat eller minskat energiintag. De fungerar genom att binda till styrregioner i gener med betydelse för inlagring eller förbränning av fett. Det finns tre olika medlemmar i PPAR-familjen; PPAR alfa (PPARA), PPAR gamma (PPARG) och PPARD delta (PPARD). PPARA finns mest i levern, PPARG i fettväv medan PPARD finns i alla kroppens celler. Det har visat sig att aktivering av PPARD med en syntetisk molekyl, ”GW501516” ökar mängden av det ”goda” HDL-kolesterolet och minskar det ”onda” LDL-kolesterolet. Syftet med denna avhandling var att identifiera gener som styrs av PPARD, samt studera hur nivåerna av PPARD styrs.

I den första studien undersöks resultatet av aktivering av PPARD i leverceller med GW501516 på apolipoprotein A-II (apoA-II) -genen. ApoA-II är ett vanligt förekommande protein i HDL-partikeln. Studien visar att PPARD kan binda till ett regulatoriskt område i apoA-II-genen och öka dess aktivitet, vilket skulle kunna tyda på att det är en av mekanismerna för hur PPARD-aktivering kan öka mängden HDL-kolesterol.

I den andra studien undersöks hur en potentiell läkemedelskandidat som binder PPARA, AZD4619, påverkar alaninaminotransferas (ALT), ett leverenzym som traditionellt används för att mäta leverskada. I en klinisk studie med AZD4619 observerades höjningar i plasmanivåerna av ALT, tydande på leverskada. Vår studie visar att aktivering av PPARA men även av PPARG och PPARD, kan styra ALT-genen och på så sätt öka mängden ALT-protein i cellerna. Slutsatsen är att ALT-höjningarna i den kliniska studien med AZD4619 inte nödvändigtvis berodde på leverskada utan kan ha orsakats av en regulatorisk effekt av PPARA.

Den tredje studien visar att nivåerna av PPARD kan styras genom alternativ splicing. För att en gen ska kunna ge upphov till ett protein krävs ett mellansteg där messenger-RNA (mRNA) bildas. För PPARD kunde vi se att det fanns olika typer av mRNA där längre varianter inte gav upphov till protein lika lätt som kortare varianter. Vi fann också en kortare PPARD proteinvariant, PPARD2, som kunde hämma funktionen av fullängdsvarianten, PPARD1. Slutsatsen är att alternativ splicing reglerar mängden av tillgängligt PPARD protein i kroppen, vilket skulle kunna ha betydelse vid exempelvis förändringar beroende på fysiologiska omständigheter i olika organ.

Den fjärde studien visar att PPARD kan styras genom regulatoriska molekyler som kallas micro-RNA (miRNA) som binder till mRNA-molekyler av PPARD och hämmar dem från att bli protein. Studien visade att miR-9 men inte miR-29 kunde hämma PPARD. Vid behandling med inflammatoriska substanser, då mängden miR-9 ökar, kunde vi se att effekten av PPARD på sina målgener minskade i monocyter, som är vita blodkroppar ansvariga för en snabb effekt av immunförsvaret vid en infektion. Vi undersökte även om det var skillnad i miR-9-mängd mellan proinflammatoriska M1- och antiinflammatoriska M2 makrofager, men såg ingen skillnad mellan dessa subtyper. Slutsatsen är att miR-9 kan ha en effekt på PPARD i monocyter men verkar ha mindre betydelse i makrofager.

Studierna i denna avhandling har visat att apoA-II- och ALT1-generna styrs av PPARD samt att mängden PPARD kan moduleras genom de regulatoriska mekanismerna alternativ splicing och miRNA-inhibering.

# LIST OF PUBLICATIONS

This thesis is based on the following original paper, which are referred to by their Roman numerals:

I. **Thulin P**, Glinghammar B, Skogsberg J, Lundell K and Ehrenborg E.

PPAR delta increases expression of the human apolipoprotein A-II gene in human liver cells

*International Journal of Molecular Medicine 2008;21(6):819-24*

II. **Thulin P**, Rafter I, Stockling K, Tomkiewicz C, Norjavaara E, Aggerbeck M, Hellmold H, Ehrenborg E, Andersson U, Cotgreave I and Glinghammar B.

PPAR alpha regulates the hepatotoxic biomarker alanine aminotransferase (ALT1) gene expression in human hepatocytes

*Toxicology and Applied Pharmacology 2008;231(1):1-9*

III. Lundell K*, **Thulin P***, Hamsten A and Ehrenborg E

Alternative splicing of human peroxisome proliferator-activated receptor delta (PPAR delta): effects on translation efficiency and trans-activation ability

*BMC Molecular Biology 2007;8:70*

* Equal contributors

IV. **Thulin P**, Werngren O, Cheung L, Fisher R, Grandér D, Corcoran M and Ehrenborg E

MicroRNA-9 regulates expression of peroxisome proliferator-activated receptor delta (PPARD) in human monocytes

*Manuscript*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABCA1 | ATP-binding cassette transporter A1 |
| ACO | acyl CoA oxidase |
| AF | activation function |
| ALT | alanine aminotransferase |
| ApoA-I | apolipoprotein A-I |
| ApoA-II | apolipoprotein A-II |
| BCL-6 | B-cell lymphoma 6 protein |
| BMI | body mass index |
| ChIP | chromatin immunoprecipitation |
| CM | chylomicron |
| CE | cholesteryl ester |
| CVD | cardiovascular disease |
| DBD | DNA-binding domain |
| DR | direct repeat |
| EMSA | electrophoretic mobility shift assay |
| FABP | fatty acid binding protein |
| FACS | fluorescence activated cell sorting |
| HDL | high density cholesterol |
| IFNγ | interferon gamma |
| IL | interleukin |
| LBD | ligand-binding domain |
| LDL | low density lipoprotein |
| LPL | lipoprotein lipase |
| LPS | lipopolysackaride |
| M-CSF | macrophage colony-stimulating factor |
| miR | microRNA |
| MMP | matrix metalloproteinase |
| NCoR | nuclear receptor corepressor |
| NR | nuclear receptor |
| PCR | polymerase chain reaction |
| PPAR | peroxisome proliferator-activated receptor |
| PPRE | PPAR response element |
| PUFA | polyunsaturated fatty acid |
| RACE | rapid amplification of cDNA ends |
| RISC | RNA-induced silencing complex |
| ROS | reactive oxygen species |
| RXR | retinoid X receptor |
| SMART | silencing mediator for retinoic acid and thyroid hormone receptor |
| SPPARM | selective PPAR modulators |
| SUMO | small ubiquitin-like modifiers |
| TG | triglyceride |
| TNFα | tumour necrosis factor alpha |
| ULN | upper level of normal |
| UTR | untranslated region |
| VLDL | very low density lipoprotein |

# PREFACE

In this thesis I will introduce the research field of cardiovascular disease and the importance of the regulation and activation of the nuclear receptor PPARD. However, before doing so I would like to explain my reasons for choosing this particular topic. Ever since I was a child I have enjoyed running and jumping, and for many years I participated in track and field events. Naturally I became fascinated by physiology and the human body, so after graduating from Upper Secondary School, I studied "Integrative Human Medicine" for one year at Karolinska Institutet. In this time the topic of transcription factors was introduced to me and during a ten-week literature study about the AhR nuclear translocator (ARNT), my interest for research and transcription factors grew. A few years later, during my Master's in Biomedicine I was introduced to the nuclear receptor PPARD in a lecture given by Associate Professor Ewa Ehrenborg. I was fascinated by the concept of a transcription factor that had important functions in metabolism but that could also be modulated by exercise. I was very happy when Ewa accepted me as a summer student in her group and after finishing my Master's thesis on PPARD I was offered a PhD position and could not resist the opportunity.

# 1 INTRODUCTION

## 1.1 CARDIOVASCULAR DISEASE

Cardiovascular diseases (CVD) include a group of disorders of the heart and blood vessels which constitutes the major cause of death in the Western world today [1]. Myocardial infarction (MI) and stroke are the main killers of this group, although peripheral vascular disease also plays a role. The main risk factors for CVD are genetic predisposition and smoking together with the components of the metabolic syndrome, which include hypertension, dyslipidaemia, obesity (abdominal) and decreased glucose tolerance (i.e. insulin resistance) (Fig. 1) [2]. The world-wide increase in obesity is a major contributor to the increasing prevalence of the metabolic syndrome and the changes in our life-styles that have occurred during the last decade are to blame [3]. The access to high-caloric food and drinks has increased in combination with sedentary occupations and leisure. In the US about 68 % of the population is reported to be overweight (BMI >25) and 34 % are obese (BMI >30) compared to 45 % and 13.5 % fifty years earlier. The corresponding numbers in Sweden today is 45 % overweight and 12 % obese, respectively [4]. Accordingly, the numbers of people developing type-2 diabetes are increasing throughout the world and these individuals are at higher risk of developing CVD. The fact that the prevalence of obesity and type-2 diabetes is increasing in children and adolescents is also alarming, both from a socioeconomic and public health point of view [5].

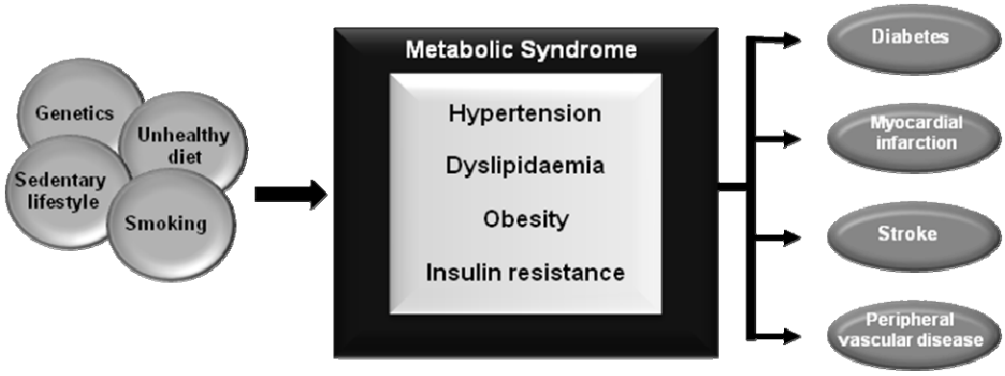


**Figure 1**. **The central role of the metabolic syndrome in vascular disease**
Multiple environmental and genetic factors contribute to the formation of the metabolic syndrome marked by hypertension, dyslipidemia, obesity and insulin resistance. These risk factors in turn contribute to initiation and progression of type-2 diabetes and the macrovascular diseases, i.e. myocardial infarction, stroke, and peripheral vascular disease. *(Adapted from Razani et al. 2008)*

### 1.1.1 Lipoprotein metabolism

To understand the mechanisms for development of CVD, a brief introduction to the metabolism of lipids in the body is necessary. Dietary lipids are transported between the tissues of the body in the bloodstream as lipoprotein particles (Fig. 2) [6], which consist of a core of lipid surrounded by a shell of phospholipids and apolipoprotein, proteins specific for the destination of the lipoprotein particles. The lipoprotein particles include the chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) and they all have specific compositions of lipids and apolipoproteins [7]. The lipoprotein metabolism is involved in determining the concentration of lipids in plasma and thus influences the amount of fat accumulation in tissues and arteries over a life span. Imbalance between production and removal of plasma lipids affects the homeostasis of the system and results in diseases caused by arterial lipid accumulation, such as atherosclerosis. Genes encoding for transcription factors that regulate proteins involved in the lipoprotein metabolism are central for the homeostasis of this system [8].

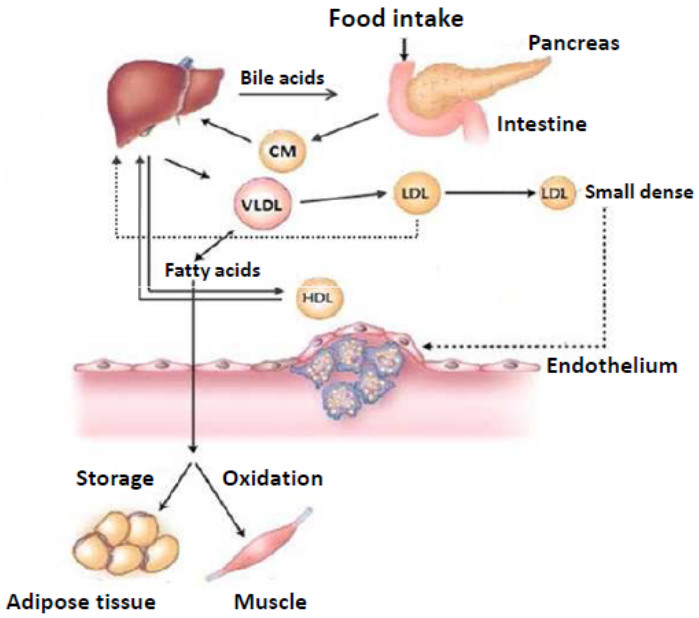


**Figure 2. Schematic overview of the lipoprotein metabolism**
Simplified overview of the lipoprotein metabolism showing the major lipoproteins and their routes. See text for details. *(Adapted from Enkhmaa et al. 2010)*

Dietary lipids are degraded by pancreatic enzyme, subsequently absorbed by the intestinal mucosa cells and packed into the CMs. The CMs mainly contain triglycerides (TG) and some cholesteryl esters (CE) in the lipid core, and the apolipoprotein B48 in the outer shell. The CMs are released into the blood stream and the TGs in the particles are hydrolyzed into free fatty acids by the enzyme lipoprotein lipase (LPL), which is

located on the surface of the endothelium in the tissues. The fatty acids are taken up primarily by skeletal muscle and adipose tissue and the CM remnants, which are enriched in CEs, are recycled by the liver. The liver produces and secretes VLDL particles, which consist primarily of TGs but also CEs, and they are labeled with the apolipoprotein B100. The function of VLDL is to provide the peripheral tissues with lipids, just like the CMs. As the VLDLs move through the circulation, they are depleted of triglycerides and enriched in cholesterol and become so called LDL particles. The LDL particles provide the tissues with cholesterol and are considered to be atherogenic. On the contrary, the HDL particles are responsible for the reverse cholesterol transport which means that HDL delivers cholesterol back to the liver, where it can be further metabolized and excreted as bile. The HDL particles are synthesized by the liver and they mainly contain the apolipoproteins A-I and A-II. These particles can interact with cholesterol transporters on the surface of cells in the periphery; ABCA1 in macrophages and ABCG1 in endothelial cells, which loads them with cholesterol. As the HDL particles return to the liver they are captured by the scavenger receptor B, class 1 receptors (SRB1) [9]. The antiatherogenic function of apoA-I is established, whereas the role of apoA-II has been more debated [10]. The effects of PPARD activation on apoA-II expression will be investigated in paper I.

### 1.1.2 Atherosclerosis

Atherosclerosis is the underlying reason for development of CVD. It is a process resulting in the formation of a lipid-rich plaque in large and medium-sized elastic and muscular arteries, which in case of rupture causes sudden thrombotic occlusion of the artery at the site of disruption (Fig. 3) [11]. In the heart, atherosclerosis can lead to myocardial infarction, whereas in the arteries of the brain, it can cause ischaemic stroke. Furthermore, atherosclerotic lesions in other arterial branches, might result in renal impairment, hypertension and critical limb ischaemia [12, 13]. The pathophysiology of atherosclerosis in not totally understood, but it involves the formation of lesions in the arteries which are characterized by lipid accumulation, inflammation, cell death and fibrosis. The fatty streaks are the earliest signs of lesions and they are present in the arteries already early in life and with increased age these fatty streaks might develop into atherosclerotic plaques [14]. A major risk factor to develop CVD is dyslipidaemia, characterized by elevated triglyceride levels, high levels of circulating LDL cholesterol, and low levels of HDL cholesterol [15]. The LDL cholesterol, especially the small dense LDL particles [16], accumulate in the

intima of the arterial vessels where they are oxidized, which induces endothelial expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) [17, 18]. These molecules attract monocytes from the blood stream to enter the intimal part of the artery, where they differentiate into macrophages under the influence of macrophage colony-stimulating factor (M-CSF) produced by endothelial cells and smooth muscle cells [19], and start expressing scavenger receptors [20]. The monocytes and macrophages belong to the innate immunity, the first line of defense of the body, and the scavenger receptors on macrophages detect oxidized LDL and promote their phagocytosis. As the disease progresses, the macrophages are overloaded with cholesterol, and turn into foam cells that start to produce inflammatory molecules which initiate an inflammatory response of the adaptive immunity, recruiting T-cells into the plaque. Activated T-cells produce inflammatory cytokines including interferon γ (IFNγ) and tumour necrosis factor α (TNFα), which leads to further activation of macrophages and endothelial cells [12]. The activated macrophages produce additional IFNγ and TNFα, as well as matrix-degrading enzymes, matrix metalloproteinases (MMPs), and reactive oxygen species (ROS). IFNγ and TNFα inhibit smooth-muscle-cell proliferation and collagen production, which increases the vulnerability of the plaque and hence the risk of rupture [21]. The MMPs further destabilize the plaque by degradation of the protective collagen and elastin [22], whereas the ROS contribute to oxidative modification of LDL and oxidative damage of DNA [23]. Furthermore, TNFα inhibits the enzyme LPL, leading to hypertriglyceridemia, which also promotes atherogenesis [24].

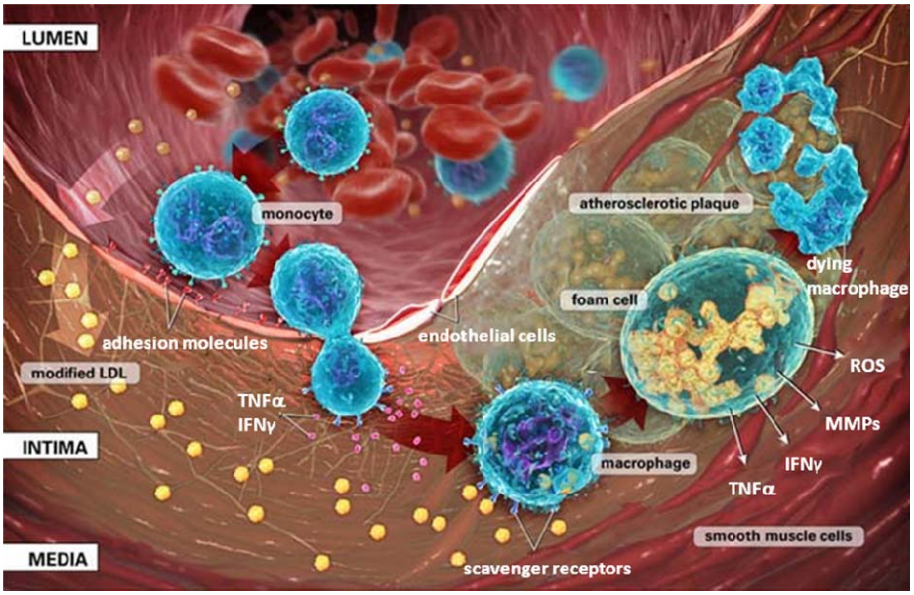


**Figure 3. Simplified illustration of the initiation and progression of the atherosclerotic plaque**
The importance of macrophages in the atherosclerotic process is emphasized. See text for details.
*(Adapted from [www.invivo.ca/illustration_and_print_formation_of_atherosclerosis.html](www.invivo.ca/illustration_and_print_formation_of_atherosclerosis.html))*

#### *1.1.2.1 Macrophages in atherosclerosis*

Recently, different macrophage subsets have been identified in the atherosclerotic lesions, which are important for the inflammatory status of the plaque. The "classically activated" M1 macrophages are induced by LPS and Th1 cytokines, mainly IFNγ, and they produce inflammatory mediators, such as IL-6 and TNFα. In contrast, the "alternatively activated" M2 macrophages develop as a response to the Th2 cytokines IL-4 and IL-13 and they secrete antiinflammatory mediators, such as transforming growth factor beta (TGFβ) and IL-10, to dampen the inflammatory response [25]. Both macrophage subpopulations are expressed in atherosclerotic lesions and modulate the inflammatory response, thereby they might have an impact on plaque stability and hence risk of development of CVD [26]. However, there is a plasticity between the subtypes and activated M1 macrophages can be modulated into M2 macrophages by expression of Th2 cytokines and vice versa [27]. It has been shown that expression levels of PPARG in atherosclerotic lesions are correlated with M2 markers, suggesting that PPARG plays a beneficial role in atherosclerosis by regulating macrophage subclasses. Also, PPARG activation primes human primary monocytes into the alternatively activated phenotype [26]. Accordingly, myeloid specific deletion of PPARG in mice impairs alternative activation and predisposes these animals to development of diet-induced obesity, insulin resistance, and glucose intolerance [28]. Additionally, the PPAR family member PPARD might be a major determinant of macrophage subtype activation as its expression is essential for alternative activation of resident macrophages in the liver and adipose tissue [29, 30]. Loss of PPARD expression in the macrophages in these tissues results in insulin resistance and obesity, respectively. However, a recent report did not show any correlation between PPARD mRNA expression and M2 markers in human atherosclerotic lesions [31] and this subject will be further investigated in paper IV.

### 1.1.3 Drugs in the treatment of CVD

#### *1.1.3.1 Drugs in the treatment of dyslipidaemia*

It has been concluded that the most important and cost-effective interventions for the atherogenic lipid phenotype are an appropriate diet, weight loss, exercise, and smoking cessation [32]. In addition, there are pharmacological agents that can improve the metabolic profiles in already affected individuals. Most of the drugs in use today have effects on lowering of LDL cholesterol, since it has been considered the most

important parameter of the atherogenic dyslipidaemia [33]. However, low plasma HDL cholesterol has also been recognized as an independent risk factor of CVD and should be treated in addition to the LDL cholesterol [34-36]. Statins are the drugs of first choice for patients with high plasma concentrations of cholesterol commonly observed in dyslipidaemia, CVD, type-2 diabetes and other high risk states of atherosclerosis. Statins are primarily LDL cholesterol lowering agents and inhibit hydroxymethylglutaryl-CoA reductase, which catalyses the rate-limiting step in the endogenous cholesterol synthesis [33]. However, there are compounds which can be used as alternative drugs if statins are not well tolerated or in combination with statins if the desired effect is not achieved. The fibrates are drugs that activate PPARA in muscle, liver, and other tissues. They mainly decrease hepatic VLDL secretion and therefore plasma triglycerides, but fibrates also decrease the levels of LDL cholesterol in individuals with elevated baseline plasma LDL concentrations and increase HDL cholesterol levels in patients with low baseline plasma concentrations of HDL [37, 38]. Nicotinic acid (also known as Niacin) decreases both LDL cholesterol and triglycerides and it is the strongest HDL raising drug in clinical use at the moment. However, the main limitation for the use of nicotinic acid is its low tolerability, as it causes flush in almost every patient [33]. In addition the anion exchange resins, cholesterol absorption inhibitors and omega-3-fatty-acids are all compounds that can be used as combination therapies together with statins, however the effects on cardiovascular end-points are not established for the two latter ones. Large ongoing trials address the decisive question whether treatment with fibrates and niacin provides additional cardiovascular risk reduction when given in addition to statin treatment [33].

#### *1.1.3.2 Drugs in the treatment of type-2 diabetes*

In order to control glucose levels in type-2 diabetes patients, the first line of drugs have been metformin and sulfonylureas. Sulfonylureas increase the secretion of insulin from the pancreatic beta cells [39] whereas metformin has been identified as an activator of AMP kinase, reducing glucose output from the liver and increasing the peripheral glucose utilization, in addition to its direct vascular effects [40]. The thiazolidinediones (TZDs) or glitazones are PPARG agonists which increase insulin sensitivity due to the uptake of free fatty acids from the circulation by the adipose tissue [41]. In addition there are some new insulin sensitizing drugs out on the market, which will not be discussed in this thesis.

### *1.1.3.3 Hepatotoxicity*

One of the most common reasons for stopping the development of promising drug candidates is hepatotoxicity discovered during preclinical studies. Furthermore, nearly 50% of all drugs fail in the post-marketing phase due to unexpected toxicity or unwanted interference with metabolism issues [42]. Since the introduction into clinical monitoring some 50 years ago, serum alanine aminotransferase (ALT) has become the standard biomarker for detection of liver injury [43]. ALT activity has been detected mostly in liver tissue [44] and ALT in serum is believed to be due to leakage from damaged hepatocytes. Serum aspartate aminotransferase (AST) activity is considered a less specific biomarker of liver function compared to ALT, since AST is present to a higher degree in skeletal and cardiac myocytes than in hepatocytes [45]. Elevations in ALT activity show high correlations to liver damage caused by drug toxicity, infection, alcohol, cirrhosis and inflammatory steatosis, and ALT activity is usually between 10-100 times higher than normal levels in these states [46]. However, the function of ALT as a perfect liver injury marker has recently been questioned [47]. While it is true that the liver is the most ALT rich organ, ALT is also found at high concentrations in kidney, cardiac and skeletal muscle [48]. Furthermore, ALT is an important enzyme for gluconeogenesis and amino acid metabolism in humans (Fig. 4) [49]. Approximately 10% of the patients taking fenofibrate show increased aminotransaminases above normal levels. Despite this, there has been no reports of hepatic pathologies with fenofibrate use [50, 51] and in 1998, Edgar et al suggested that fibrates could increase the gene expression of ALT as an alternative "non-toxic" mechanism for the elevation of ALT enzymes in serum [46]. The normal turnover of hepatocytes, releasing their increased amounts of intracellular aminotransferases, would be the reason for the mild increase in serum ALT observed after treatment with fibrates. The subject of a possible PPAR-mediated regulation of the ALT1 gene will be investigated in paper II in this thesis.

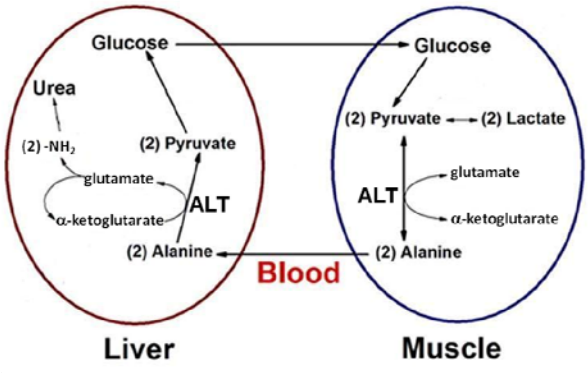


**Figure 4. Schematic overview of the glucose-alanine cycle catalysed by the enzyme ALT**
ALT is responsible for transamination of amino acids in the muscle and for gluconeogenesis in the liver. The amino groups transported from the muscle to the liver are converted into urea which is excreted in the urine. *(Adapted from www.themedicalbiochemistrypage.org)*

## 1.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors (NRs) which contain transcription factors that regulate the expressions of genes involved in processes such as reproduction, development and general metabolism. There are 48 members of the NR superfamily in humans and 49 in the mouse [52] and they all share a common structure. The PPARs are classified as members of group C in the nuclear receptor subfamily 1 (NR1C) [53] and they belong to a functional subgroup of the nuclear receptors denoted adopted orphan receptors (Fig. 5) [54].

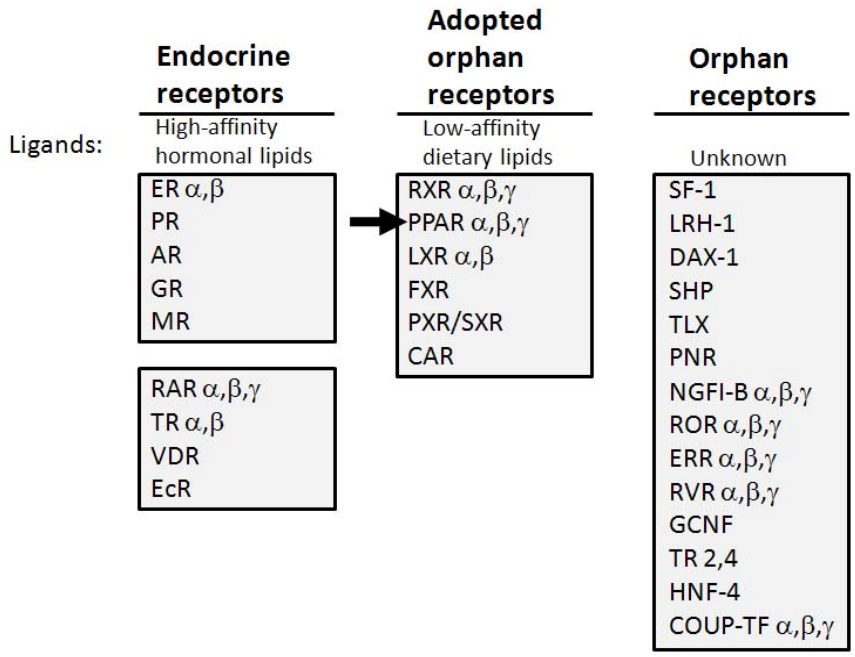


**Figure 5. Functional grouping of the nuclear receptor superfamily**
The PPAR subfamily belongs to the group of adopted orphan receptors and is indicated in the figure with an arrow. *(Adapted from Chawla et al 2001)*

PPARA (NR1C1) was the first member of the PPAR family to be discovered in the early 1990s [55] and obtained its name from the fact that it could bind agents that caused peroxisome proliferation in rodents. PPARB (NR1C2) and PPARG (NR1C3) were identified a few years later in Xenopus [56] and subsequently murine PPARG and one more paralogue which was named PPARD were identified [57]. However, when the chicken PPARB was cloned and characterized, it was evident that murine PPARD was the ortholog of xenopus PPARB and hence this receptor is now referred to as PPARB/D. In this thesis it will exclusively be referred to as PPARD. The PPARs are

expressed by separate genes on different chromosomes and they are expressed in a tissue-specific manner.

### 1.2.1 Structure

All the nuclear receptors share a common structure which makes them able to bind to DNA and exert their functions as regulators of gene transcription (Fig. 6) [54]. The N-terminal ligand-independent activating function 1 (AF-1) domain is the most variable between the PPAR family members, and it is recognized by coactivators and is considered to be important for transactivation and transcription [58]. The DNA-binding domain (DBD) is very well conserved and it is constituted by two highly conserved zinc finger-like structures which are responsible for receptor binding to its specific DNA sequences, the PPAR response elements (PPRE), and it also plays a role in dimerization [59]. The hinge region (H) permits protein flexibility to allow simultaneous receptor dimerization and DNA binding [54]. The ligand binding domain (LBD) consists of 12 α-helices (H1-12) which together constitute a pocket for the ligand. The entrance to the pocket is guarded by H12, which contains residues that are crucial for the function of AF2. In the absence of ligand, corepressors are bound to H3 and H4 but when a ligand is bound, H12 induces a conformational change in the receptor and the corepressors are released. Instead, coactivators which contain LxxLL-like motifs (where L is leucine and x is any amino acid) can bind to a hydrophobic cleft formed by H3, H4 and H12 which supports coactivator interaction and gene transcription. The LBD is also essential for dimerization. [60]

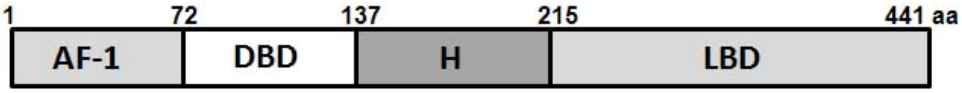


**Figure 6. Schematic representation of the functional domains of PPARD**
The N-terminal domain contains the activation function 1 (AF-1). DBD = DNA-binding domain, H = hinge region, LBD = ligand-binding domain. Amino acid (aa) numbers are shown above the receptor. *(Adapted from Ehrenborg and Krook 2009)*

### 1.2.2 Cofactors

The nuclear receptors need additional proteins called cofactors to be able to exert their functions. The nuclear receptor cofactors include corepressors, coactivators and their associated proteins, and over 300 of them have been identified [61]. The coactivators directly bind to transcription factors and positively regulate target gene

transcription, either by modification of histone and chromatin structure to open up DNA for transcription, while others provide linkage to the core basal transcriptional machinery. CBP/P300 and SRC-1 are examples of coactivators which have been shown to associate with the PPARs and they loosen up the chromatin structure to enable transcription by possessing histone acetylace transferase (HAT) activity [62-65]. The corepressors inhibit target gene transcription by recruitment of histone deacetylases (HDACs) to enforce a tight chromatin structure and classical examples are NCoR and SMRT [66, 67]. As far as is known, there are no receptor specific coactivators that direct the overall transcriptional activity of particular members of the PPAR subfamily [68]. In the absence of ligand, the PPARs are maintained in the nucleus in a repressed state by nuclear receptor corepressors such as NCoR and SMRT [69-71]. PPARD is the only PPAR subtype which is able to bind DNA when bound to corepressors and it can thereby repress PPAR target genes in the absence of ligand [69, 70, 72]. Another mechanism of gene repression exerted by PPARD is its association with BCL-6, which functions as a corepressor for genes involved in inflammation [73]. In the unliganded state, PPARD binds to and sequestrates B-cell lymphoma 6 protein (BCL-6), whereas ligand binding causes BCL-6 dissociation and repression of inflammatory genes. Binding of a ligand to a PPAR results in the dissociation of the corepressors and then an orchestrated recruitment of several transcriptional coactivators which facilitates gene transcription. Besides ligand binding, activation or repression of target genes by the PPARs also depends on the cellular expression pattern of coactivators and corepressors, as has been taken advantage of in the SPPARM concept (described in section 1.2.6.2) [74].

### 1.2.3 Function

The PPARS are lipid sensors with distinct but overlapping functions in glucose and lipid metabolism. The natural ligands for all the PPARs are unsaturated fatty acids and their derivatives. Upon ligand binding the PPARs heterodimerize with the retinoid X receptor (RXR) and bind to PPRE which consists of a direct repeat (DR) of the consensus sequence AGGTCA separated by one nucleotide, called a DR1 motif [75] (Fig. 7) [58]. It has been reported that the 5'-flanking region to the PPRE is responsible for subtype specific activation of the PPARs [76]. The conformational change induced by ligand activation releases corepressors and allows coactivators to bind, which induces transactivation and transcription.

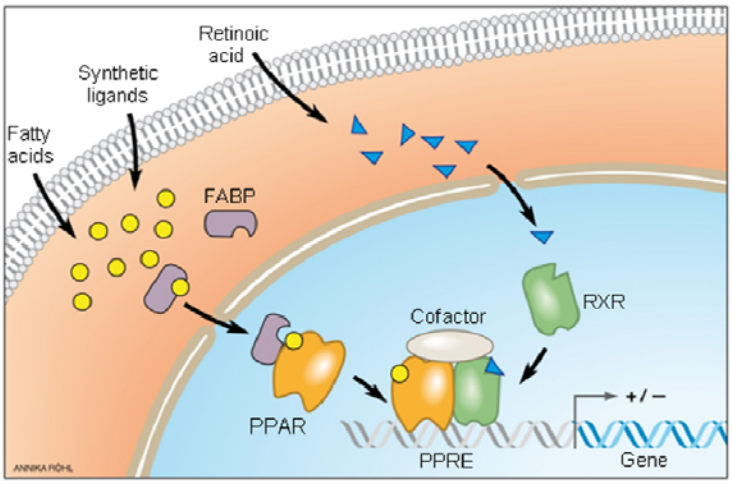


**Figure 7. PPAR ligand binding, dimerization and transactivation**
Upon ligand binding the PPARs form heterodimers with RXR and bind to PPRE in gene promoters. The effects are further modified by interaction with specific cofactors. Fatty acid binding proteins (FABP) may bind and deliver ligands to the receptor. *(Adapted from Ehrenborg and Krook, 2009)*

### 1.2.4 PPARA

The human *PPARA* gene consists of eight exons and it is localized at chromosomal region 22q12-q13.1 [77, 78]. PPARA is primarily expressed in tissues with a high level of fatty acid metabolism such as liver, skeletal muscle, heart, kidney, and intestine [79]. The major role of PPARA is the regulation of energy homeostasis and mainly in the liver, PPARA activates fatty acid catabolism, stimulates gluconeogenesis and ketone body synthesis [80, 81]. The function of PPARA in hepatic fatty acid metabolism is especially prominent during fasting and in normal mice, PPARA expression increases during fasting. In contrast, fasted PPARA knock-out mice have fatty livers and elevated levels of free fatty acids in plasma due to the inability of the liver to oxidize the fatty acids released from adipose tissue [81]. Furthermore, fasted PPARA null mice show decreased levels of plasma glucose and ketone bodies due to decreased gluconeogenesis and lipid oxidation, respectively. In addition, the starved PPARA mice suffer from hypothermia [81]. An additional function of PPARA is to control the hepatic expression of enzymes involved in the control of amino acid metabolism and urea synthesis [82]. PPARA is also involved in the regulation of lipoprotein assembly in the liver, which is demonstrated by the capacity of the PPARA activating fibrates to increase the expression of apolipoproteins involved in the reverse cholesterol transport [83-85]. PPARA has also been described to be a modulator of inflammation by negative crosstalk with the NFκB pathway and treatment of hyperlipidemic patients with fibrates is shown to decrease the elevated levels of TNFα and IFNγ [86-88].

### 1.2.5 PPARG

The human *PPARG* has nine exons and is located on chromosome 3p25 [89]. The human PPARG gene gives rise to three different transcripts due to alternative promoter usage (PPARG1-3) but after alternative splicing of the 5' -end of the transcripts, PPARG1 and 3 gives rise to the same protein, hence there are two protein isoforms of the human PPARG (PPARG1 and PPARG2) [90]. The functional difference between the isoforms is not clearly understood but PPARG2 expression is restricted to adipose tissue where it seems to play a major role in adipocyte differentiation, whereas PPARG1 is more widely expressed. Adipose tissue, large intestine, and hematopoietic cells express the highest amounts of PPARG1 while kidney, liver, and small intestine have intermediate levels. PPARG3 is abundant in macrophages, the large intestine and white adipose tissue [90].The function of PPARG is to induce adipocyte differentiation and increase the expression of genes involved in lipogenesis [91]. Furthermore, PPARG is involved in the glucose metabolism through an improvement of insulin sensitivity, when fatty acids are taken up from the circulation. Lately PPARG has also been recognized to play an important role in inflammation, favouring a more antinflammatory phenotype of the residential macrophages in adipose tissue and liver [28].

### 1.2.6 PPARD

Human *PPARD* is located at chromosomal region 6p21.2-p21.1 and comprises nine exons [92]. PPARD is ubiquitously expressed and its function was an enigma for a long time. It is the least studied of the three PPAR subtypes due to its ubiquitous expression and unavailability of selective ligands [93]. However, during the last decade it has received more attention and its role in energy homeostasis and fatty acid catabolism has been recognized since the specific ligand GW501516 was synthesized. Administration of GW501516 to mice increases oxidation of fatty acids in the skeletal muscle and ameliorates diet-induced obesity and insulin resistance [94]. In insulin resistant rhesus monkeys, treatment with GW501516 showed decreased plasma triglyceride and LDL cholesterol levels, especially the small dense LDL particles, as well as increased plasma HDL levels. Additionally, the insulin levels of the monkeys were decreased by treatment with the PPARD agonist [95]. The importance of PPARD for lipid metabolism has also been demonstrated by transgenic tissue-specific expression in mice. Transgenic expression of an activated form of PPARD in adipose tissue results in lean mice which are resistant to obesity induced by a high-fat diet

[96]. In parallel, muscle-specific PPARD overexpression results in a shift to more oxidative muscle fibers (type 1), increased expression of genes involved in oxidative metabolism and reduced body fat mass [97]. Also, endurance exercise promotes an accumulation of PPARD protein in muscle of wild-type mice and transgenic mice overexpressing the activated form of PPARD in skeletal muscle, are lean and show increased endurance and expresson of oxidative fibers compared to their wild-type littermates [97, 98]. The beneficial role of PPARD on lipid metabolism makes it a plausible target for treatment of obesity and insulin resistance. Studies in mice also reveal the importance of PPARD in the embryonic development, in the skin, brain and in the regulation of inflammatory macrophages in liver and adipose tissue [29, 30, 99, 100].

### 1.2.7 Ligands

#### *1.2.7.1 Endogenous ligands*

All the PPARs have wide ligand-binding pockets compared to the other members of the nuclear receptor family, which makes them able to bind fatty acids and their derivatives. Hence, the natural ligands for all the PPARs consist of polyunsaturated fatty acids (PUFAs), such as linoleic, linolenic and arachidoinc acid [101]. However, there are some differences in ligand preference between them; the ligand binding cavity in PPARA is more hydrophobic than the other two PPARs, which explains why it also can accommodate saturated fatty acids. In contrast, PPARD is shown to have a narrower pocket compared to PPARA and PPARG, which explains why it is more restrictive in binding saturated fatty acids [102]. Furthermore, some eicosanoids can serve as subtype specific ligands, for example leukotriene B4 can activate PPARA whereas 15-Deoxy-D12,14-PGJ2 has been identified as a more specific ligand for PPARG [101]. In contrast, the fatty acid erucic acid (C22:1), which is a weak ligand, appears more selective for PPARD [103]. In addition to ligand specificity, there are intracellular lipid binding proteins, so called fatty acid binding proteins (FABP) which bind ligands specific for the different PPAR isoforms and bring them into the nucleus of the cell where the PPARs are located. Interestingly, although FABP4 and FABP5 bind multiple ligands, only particular compounds trigger their nuclear translocation [104]. FABP4 moves into the nucleus in response to ligands that activate PPARG but not upon treatment with PPARD ligands. In contrast, FABP5 mobilizes to the nucleus only in response to ligands that activate PPARD [105].

#### *1.2.7.2 Synthetic ligands*

Because of their central role in the regulation of genes involved in energy homeostasis and inflammation, PPARs have become attractive pharmaceutical targets for the treatment of metabolic disease. The fibrates activate PPARA and the use of fibrates in the management of lipoprotein disorders has a history dating back to the mid-1960s [106]. They show few significant side-effects and are considered to be relatively safe, but have been associated with muscle weakness, myopathy and in some rare cases rhabdomyolysis [107]. The glitazones are synthetic ligands for PPARG and were introduced on the market at the end of the 1990s and they are associated with weight-gain, fluid-retention and an increased risk of bone fracture [108-110]. Since the withdrawal of Troglitazone in the year 2000 due to hepatotoxicity [111] and now the recent withdrawal of Rosiglitazone from the market in 2010 due to the increased risk of MI, stroke and overall mortality [112, 113], the safety concerns of PPARG agonists have been raised. Regarding PPARD specific agents, no compounds are in clinical use yet, but there are some small recent human studies that have evaluated the short-term effects of GW501516 [114, 115]. Both studies showed decreased triglyceride levels after two weeks treatment despite the fact that the participants were healthy and only moderately obese. Increased HDL-levels was only obtained in one of the studies [114] whereas decreased LDL cholesterol and increased insulin sensitivity was observed in the other one [115]. However, GlaxoSmithKline, the company in charge of GW501516, terminated a phase I trial with this compound in 2005 and it is no longer on their list of compounds in the pipeline [116]. Another synthetic PPARD agonist, MBX-8025, from the company Metabolex showed decreased triglyceride and LDL cholesterol and increased HDL cholesterol in an eight week long study including 173 obese or overweight participants [117]. Considering the side-effects of some PPARG agonists, which have been out on the market for years, there is already reluctance towards PPAR compounds and it is obvious that any drug acting via PPARs needs to demonstrate a favorable cardiovascular risk-benefit profile. Dual PPARA/G agonists and pan-PPARA/G/D agonists have been developed in hopes of achieving multiple therapeutic benefits but reducing the side-effects [118]. However, the safety is still an issue for these agents. Another type of second generation PPAR compound is the selective PPAR modulators (SPPARMs) which are designed to activate the PPARs in a tissue-selective manner [74]. Hence, only desirable effects of PPAR activation are

achieved whereas side-effects can be avoided. The SPPARMs bind to the PPAR and induce a conformational change in the LBD distinct from when a full agonist is binding, resulting in preferential binding of specific cofactors or corepressors. Thus, differential PPAR effects can be achieved depending on tissue expression of distinct sets of coactivators and corepressors. The SPPARγMs show good efficacy and less side-effects in pre-clinical animal models of metabolic disease and it remains to see how well they perform in clinical trials [74].

## 1.3 PPAR REGULATION

So far, the main focus of PPAR research has been to unravel the effects of activation of PPARs whereas considerably less is known about the regulation of their expression [119]. Most research has been performed using mice models, which means that the regulatory mechanisms between humans and mice might deviate. However, keeping that in mind, the main mechanisms of regulation of the PPARs will be summarized below and an overview of these mechanisms is shown in Figure 8.

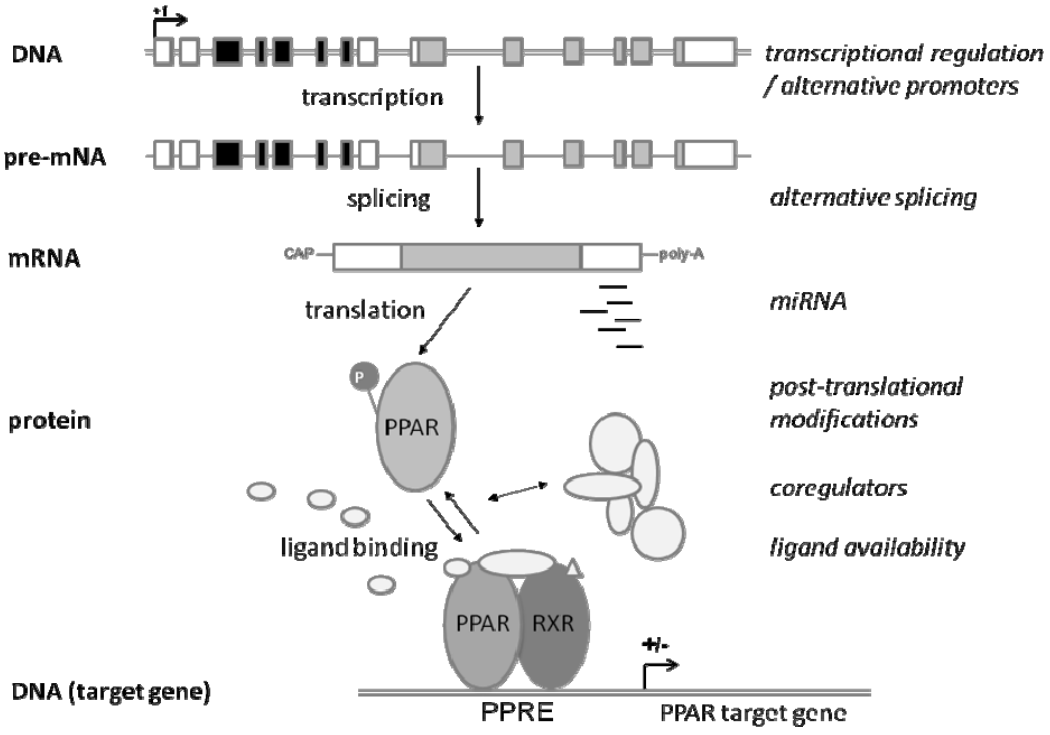


**Figure 8. Schematic overview of the main mechanisms of PPAR regulation**
The levels of regulation are depicted on the left hand side and the mechanisms of regulation are described on the right hand side of the figure. The figure is based on the PPARD gene, since it is the main focus of this thesis, even though the mechanisms are valid for all the PPAR family members. The transcriptional start of the PPAR gene is indicated with an arrow and +1.

### 1.3.1 Transcriptional regulation

Since there is not much data regarding the transcriptional regulation of the PPARs, the main focus of this chapter will be on the posttranscriptional regulation of the PPARs. However, it can be mentioned that a recent study identified differential binding of estrogen-related receptor (ERR) α and γ to a site encompassing a single nucleotide polymorphism (SNP) in the human PPARA promoter, which influences the transcriptional activity of the gene [120]. Furthermore, adipogenic hormones such as insulin and glucocorticoids induce PPARG transcription during early adipogenesis through the CCAAT enhancer-binding proteins [121]. Also, the SREBP1 and -2 directly control the expression of the human PPARG gene [122]. *In silico* analysis of the human PPARD promoter showed that it lacks a TATA box but is rich in potential Sp-1 binding elements, which is a typical feature for house-keeping genes, and it also contains two putative NFκB elements [92].

### 1.3.2 Post-transcriptional regulation

#### *1.3.2.1 Alternative splicing*

Splicing of precursor mRNA (pre-mRNA) is a crucial regulatory step in the pathway of gene expression. The mature mRNA is formed as the introns of the pre-mRNA are removed and the exons are ligated together (Fig 9) [123]. Splicing occurs in organisms ranging from yeast to human and take place within the spliceosome, a large protein complex containing the small nuclear RNAs (snRNAs) U1, U2, U4, U5 and U6, and more than 100 core proteins [124, 125] (Fig 9B) [123].

Four signals located at the exon-intron boundaries have a well characterized role in helping directing the splicing machinery. In the 5'-end of the intron, the 5' splice site denoted by the nucleotides GU in the exon/intron boundary encompassed within a larger less conserved sequence. In the 3'-end of the intron, there is the branch point containing an adenosine, a polypyrimidine tract (PPT), followed by a terminal AG [126] (Fig. 9A). In addition, there are short conserved regions in the exons and introns which act as exonic or intronic splicing enhancers or silencers. Specific binding of splicing regulatory proteins called SR proteins to these regulatory regions assists in the positioning of the spliceosome on the appropriate splice sites [127, 128].

Alternative splicing, which includes different exons in the mRNA, results in the generation of alternative isoforms and is often tightly regulated in a cell-type- or developmental stage-specific manner [129]. There are several different types of alternative splicing events (Fig. 9C) of which exon skipping is the most common in higher vertebrates, followed by alternative 3'- and 5'-splice site (SS) selection, and intron retention [130]. Also, more complex events like mutually exclusive exons, alternative promoter usage and alternative polyadenylation give rise to alternatively spliced transcripts in vertebrates [131].

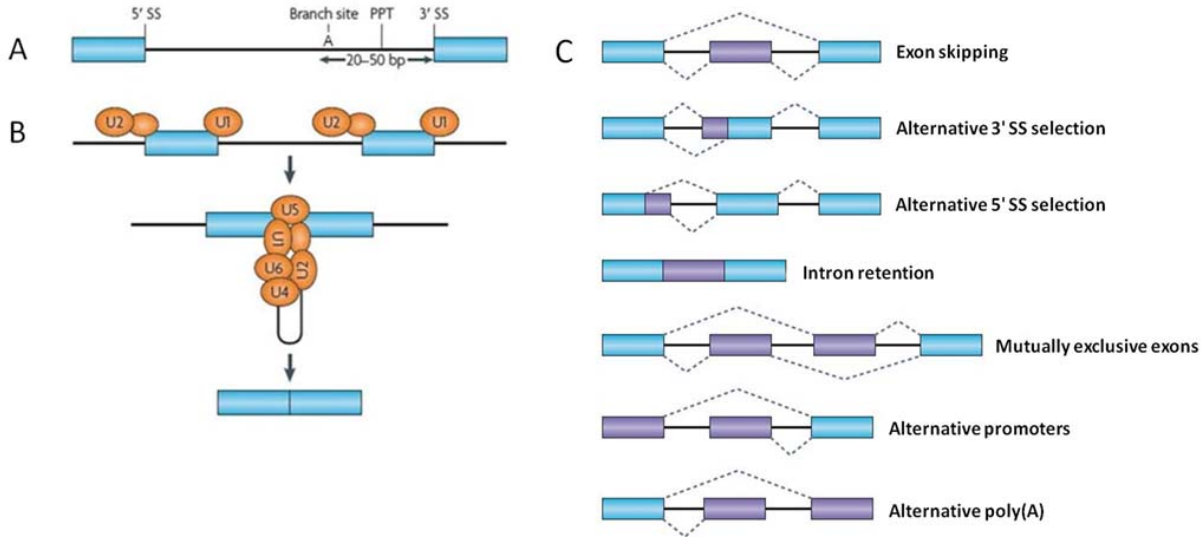


**Figure 9**. **Schematic overview of the mechanisms of splicing**
A) The four conserved signals that enable recognition of RNA by the spliceosome are shown above. B) The splicing machinery that performs the main steps in the splicing process is shown. C) Different types of alternative splicing events. SS = splice site; PPT = polypyrimidine tract. *(Adapted from Keren et al 2010)*

Dominant negative regulation by truncated receptor isoforms has been described for many of the members of the nuclear receptor superfamily, such as the glucocorticoid receptor, estrogen receptor and vitamin-D receptor [132-134]. Both the PPARA and PPARG genes have been shown to express truncated isoforms by alternative splicing, which act as dominant negative regulators of their respective full-length receptor [135, 136]. The truncated isoforms lack a part of the ligand-binding domain, which makes them unable to bind ligands, and they are expressed at low levels compared to the full-length variants. Alternative splicing for the human PPARD gene has not been studied, even though a truncated version has been reported in the NCBI database. In paper III, the putative regulation of PPARD by alternative splicing is investigated.

#### *1.3.2.2 MicroRNA*

MicroRNAs (miRNAs) are endogenous non-coding single-stranded RNAs approximately 22 nucleotides long that bind to their target mRNA and either induce mRNA degradation or inhibit protein translation [137]. They are expressed from animals to plants, are conserved between species and are implicated in diverse biological processes such as development and differentiation [138]. Furthermore, many of them have been reported to be aberrantly expressed in different forms of cancers [139]. In mammals, more than 50% of mRNAs are predicted to be the subject of miRNA-mediated control [140]. The miRNAs are initially transcribed in the nucleus bý RNA polymerase II as long primary transcripts (pri-miRNAs) which are capped and polyadenylated, and cleaved into 60-80 nucleotide hairpin structures (pre-miRNAs) (Fig. 10) [141]. After the completion of the nuclear processing, the pre-miRNA is exported into the cytoplasm by the nuclear transporter protein Exportin 5 where it undergoes further processing by the Dicer, releasing ~22 nt miRNA duplexes containing ~2 nt overhands at either end. The strand with the least stable pairing in the 5'-end (the guide strand) is selectively loaded into the RNA-induced silencing complex (RISC) which targets transcripts through either mRNA cleavage or translational repression [142, 143]. The other strand (passenger strand or miRNA*) is degraded.

The miRNAs:RISC complexes bind to and regulate their target mRNAs by partial complementary binding in the 3'- untranslated region (3'-UTR) of the mRNA. However, there are exceptions to this rule and some miRNAs have been reported to bind to the 5'-UTR or to the coding region of the mRNA [144-147]. Additional features of the target mRNA has shown to be of significance such as AU-rich elements and positioning of the sites within the 3'-UTR [148]. The most important part of binding is between nucleotide 2-8 from the 5'-end of the miRNA, the so called seed sequence [149, 150]. In addition, complementarity in the 3' end of the miRNA has proven to be of significance for increased stability of the binding and compensation of a weak seed region [150]. Another recently discovered group of miRNAs are the ones with centered pairing, which lack both perfect seed pairing and 3'-compensatory pairing and instead have 11–12 contiguous Watson-Crick pairs to the center of the miRNA [151].

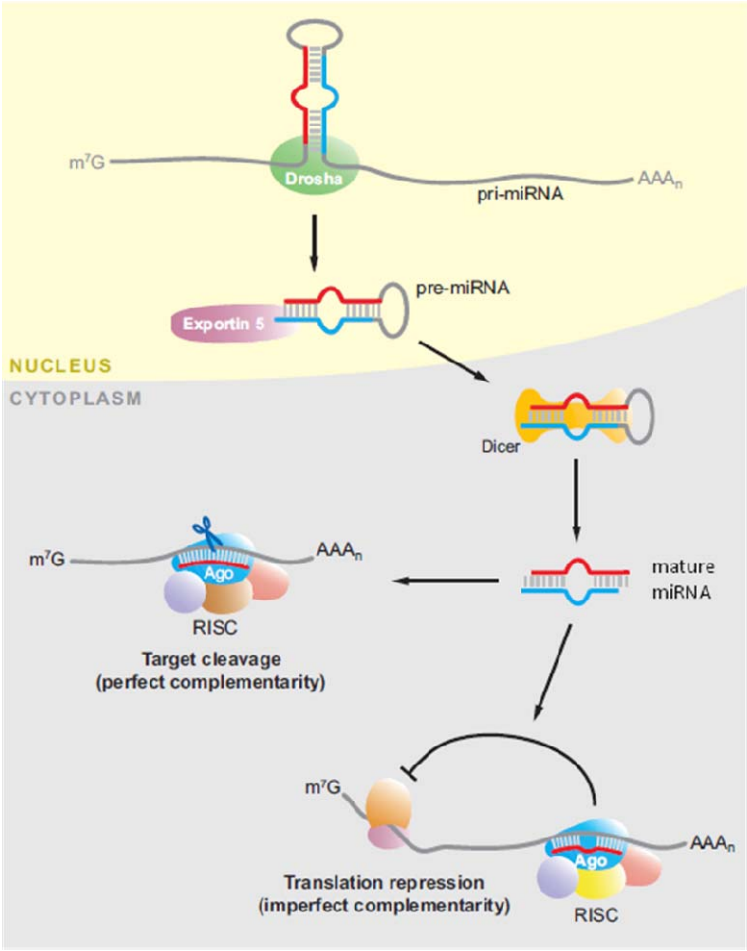


**Figure 10. Biogenesis and function of miRNA**
The miRNAs are transcribed in the nucleus and delivered into the cytoplasm where they exert their effects on mRNA degradation or protein synthesis. See text for details.
*(Adapted from Chang and Mendell, 2009)*

Transcription factors are often subjected to miRNA regulation, which introduces an additional level for the cell to control its regulators. Of the human PPARs, both PPARA and PPARG have been shown to be regulated by miRNAs. PPARA protein expression is targeted by miR-519d in subcutaneous adipose, a miRNA which is shown to be highly expressed in severely obese compared to non-obese subjects [152]. MiR-519d was also shown to increase lipid accumulation during preadipocyte differentiation, indicating that it plays a role in obesity. PPARG is targeted by miR-27 and miR-130, which are downregulated during adipocyte differentiation [153-156]. No data are yet available regarding the regulation of PPARD by miRNA, however the question of whether PPARD is miRNA regulated will be investigated in paper IV.

### 1.3.3 Post-translational regulation of PPARs

#### *1.3.3.1 Phosphorylation*

Both mPPARA and mPPARG are shown to be phosphorylated by various stimuli affecting the different domains of the receptors. For example, insulin activates MAPK which activates ERK leading to phosphorylation of the A/B domain in AF-1 containing N-terminus of the receptor, resulting in release of corepressors and increase in the transactivational activity of both receptors [157]. So far, less information is available regarding the phosphorylation of the PPARD isotype, even though there are sites predicted in the protein, which implies that PPARD also might be a phosphoprotein [158]. Furthermore, both cAMP and PKA activators are shown to increase both ligand-activated as well as basal activity of PPARD [159, 160].

#### *1.3.3.2 Ubiquitination and sumoylation*

Ubiquitin is an 8 kDa protein which covalently binds to proteins to target them for degradation by the 26S proteasome. *In vitro* studies have shown that both PPARA and PPARG can be targeted for degradation by ubiquitination. Furthermore, PPARA agonists stabilize the receptor whereas the degradation of PPARG via ubiquitination is enhanced by ligand binding [161, 162]. For PPARD, *in vitro* studies show that under conditions of moderate expression, GW501516 is not significantly influencing ubiquitination or degradation of PPARD. In contrast, overexpression of PPARD via transient transfection dramatically enhances its degradation by ubiquitination and proteosomal degradation, which is inhibited upon ligand binding [163].

Small ubiquitin-like modifiers (sumo) compose a family of three 11 kDa proteins homologous to ubiquitin which can be reversibly conjugated to the lysine residues of proteins through covalent binding [164]. Many of the sumo-modified proteins identified to date are transcription factors, coactivators, or corepressors and in the majority of cases attachment of sumo appears to repress the activity of transcriptional activators [165]. So far, among the PPAR family members PPARA [166, 167] and PPARG [168] have been described to be regulated by sumoylation. Modification of PPARA by sumoylation specifically recruits the corepressor NCoR but not SMRT which might lead to differential expression of a subset of PPARA target genes. For PPARG, it is shown that ligand-dependent sumoylation directs the receptor to the promoter of the iNOS gene after inflammatory stimuli and induces its transrepression,

which explains the inhibitory effect of PPARG on some genes involved in inflammation [168].

It is obvious from this introduction that furthers studies regarding the regulation of the PPARs are needed, especially of PPARD. Furthermore, additional PPARD target genes implicated in metabolic disease remain to be identified. Clearly this receptor has its beneficial properties, even though its broad tissue expression might limit its potential as a drug target. Hence, this thesis will explore additional target genes of PPARD as well as further elucidate the mechanisms of its regulation.

# 2 HYPOTHESIS AND AIMS

**Hypothesis:**

The overall hypothesis for this thesis is that PPARD activation increases the expression of genes beneficial to prevent the development of atherosclerosis and CVD, and furthermore that the PPARD gene per se is subjected to regulation by posttranscriptional mechanisms.

**Aims:**

The general aims of this thesis were to identify human PPAR target genes and improve our understanding of the molecular mechanisms regulating the expression of human PPARD.

Specific objectives

- To characterize the mechanisms of PPARD activation on the apoA-II gene expression (paper I)
- To determine whether the ALT genes are transcriptional targets of the PPARs (paper II)
- To investigate whether PPARD is subjected to posttranscriptional regulation via alternative splicing and miRNA (paper III and IV)

# 3 MATERIALS AND METHODS

Detailed descriptions of materials and methods are given in the individual papers.

### 3.1.1 Chemicals (paper I-IV)

The synthetic PPARD agonist GW501516 was synthesized by Synthelec AB as described [169]. Fenofibric acid was a kind gift from Professor Per Eriksson, Karolinska Institutet, Sweden. Additional fenofibric acid and AZD4619 were obtained from Astra Zeneca, Mölndal, Sweden. Rosiglitazone was obtained from Cayman Chemical Company and TNFα from Calbiochem. The cytokines used in paper IV were purchased from Peprotech and LPS from Sigma.

### 3.1.2 Bioinformatic sequence analyses (paper I-IV)

In order to find putative PPREs in the hypothesized target genes apoA-II and ALT1, the MatInspector program in the Genomatix database [170], the Alibaba 2.1 [171] and Promo [172] programs were used (paper I and II). To identify sequence conservation between species the ECR browser tool [173], and ClustalW [174] were utilized (paper I and III). Furthermore, the RepeatMasker [175] was used to look for repetitive elements in the PPARD gene. In paper IV, miRNA target site prediction was performed by the UCSC genome browser [176] (human May 2004 assembly), which includes miRNA target predicitions from the databases TargetScanS [177] and PicTar [178].

### 3.1.3 Cell culture (paper I-IV)

A panel of human cell lines have been used in this thesis; the human hepatocellular carcinoma cell lines HepG2 and HuH-7, the human embryonic kidney cell line HEK293, the cervical epithelioid carcinoma cell line HeLa, and the kidney epithelial carcinoma cell line A498 were all maintained in DMEM (1 g/l glucose), containing 10 % FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 5 % $CO_2$ in air. The monocytic leukaemia cell line THP-1 was maintained in RPMI using the same conditions as described for DMEM. Cryopreserved human hepatocytes from five different donors were obtained from In Vitro Technologies and were maintained in DMEM as described for the cell lines above (paper II). Human primary monocytes were purified from blood obtained from anonymous donors and were cultured in RPMI and 10 % human AB serum or differentiated into macrophages as described in next section (paper IV).

### 3.1.4 Monocyte isolation and macrophage differentiation (paper IV)

Monocytes were purified from buffy coats obtained from unknown healthy blood donors by endotoxin-free Ficoll purification (Ficoll paque PLUS, GE Healthcare) followed by two steps of Percoll purifications (Percoll PLUS, GE Healthcare) as described in Repnik et al [179], which resulted in about 70 % CD14 positive cells, as determined by FACS analysis. The monocytes were seeded out in 6-well plates in RPMI containing 100 ng/ml M-CSF, 20% human AB-serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). After 7 days, the monocytes had differentiated into macrophages and the medium was replaced with RPMI containing 10 % hAB serum and antibiotics as above (M0). To obtain M1 macrophages, 100 ng/ml LPS and 20 ng /ml IFNγ was also added to the fresh culture medium, whereas for M2, Il-4 was added at a concentration of 20 ng/ml. After 6, 24 and 72 hours, the cells were harvested for RNA using Qiazol or saved for FACS analyses.

For the monocyte studies, the EasySep human monocyte enrichment kit (#19059, Stem Cell Technologies) was used straight after the Ficoll purification. The cells (>90 % CD14 positive cells as determined by FACS analysis) were seeded out at in 6-well plates in RPMI containing 10% human AB-serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), in the presence or absence of 100 ng/ml LPS. The cells were harvested after 8 or 24 hours, using Qiazol (Qiagen) for subsequent analysis of total RNA, including miRNA.

### 3.1.5 Flow Cytometry Analysis (paper IV)

Differentiated macrophages (M0, M1 and M2) were incubated with APC (allophycocyanin)/Cy7-conjugated antibody CD206 (BioLegend). Analyses were performed using a CyAn ADP Analyzer flow cytometer and the Summit software (Beckman Coulter).

### 3.1.6 DNA sequencing (paper I-IV)

Sequencing was performed using a Big Dye Terminator Kit and an automatic sequencer (Genetic Analyzer 3100, Applied Biosystems) to control that all cloned constructs were correctly assembled and to verify the mutations introduced after *in vitro* mutagenesis.

### 3.1.7 Plasmids and expression vectors (paper I-IV)

- **Cloning** of promoters was performed using PCR amplification, followed by subcloning into the PCR 2.1 vector (Invitrogen) and subsequent transfer into the reporter gene vector pGL3basic (Promega) (paper I-III). Likewise, approximately two kb of the PPARD 3'-UTR was cloned into the psiCHECK2 vector (Promega) for subsequent use in the miRNA reporter assays. The 36 bp constructs covering the miRNA target sites of PPARD were obtained by annealing complimentary oligonucleotides for the sites and subsequent cloning into the psiCHECK2 vector (paper IV).
- ***In vitro*** **mutagenesis** of putative PPREs or miRNA target sites was performed using oligonucleotides containing the desired mutations and a mutagenesis kit from Stratagene.
- The **expression vector** for human PPARA was a kind gift from Eleanor S Pollak, University of Pennsylvania School of Medicine, PA, USA. The expression vector for PPARD was kindly provided by Dr. CN Palmer and is described elsewhere [180]. The PPARD expression vector was modified into PPARD2 by replacement of the 3'-end of PPARD1 with a PCR-product containing the 3'-end of PPARD2 (paper III). To obtain the full-length PPARD, the whole 3'-UTR of PPARD1 was inserted into the vector coding for PPARD1 (paper IV).
- The **TNT vector** coding for PPARD was cloned as described above, using the full-length sequence for PPARD. The TNT vectors for PPARA, PPARG and RXRA were all cloned by our collaborators at Astra Zeneca.
- The **miRNA expression vectors** coding for either miR-9, miR-29 or miR-155 were created by PCR amplification of the genomic loci and subsequently cloned into the pcDNA 3.1/V5-His-TOPO, by our co-authors at CCK, Karolinska Institutet.

### 3.1.8 Transient transfection and Luciferase assays (paper I-IV)

For the reporter assays, the cells were seeded out in 24-well plates in PEST-free medium and the following day, the appropriate reporter constructs and expression vectors were added together with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For the promoter studies, agonists or other compounds were added in fresh medium after 24 hours and the cells were treated for an additional 24 hours, whereas the miRNA reporter assays were harvested after 24 hours of transfection. The cells were then lysed with a triton-X containing lysis buffer and the

luciferase activities of the lysates were measured in a luminometer (Lucy2, Anthos). For normalisation of the transfection efficiency, a β-galactosidase plasmid was cotransfected with the reporter plasmid and β-galactosidase activity was also measured (paper III). The psiCHECK2 vector used in paper IV contains the sequences coding for both renilla and firefly luciferase, hence no external normalisation plasmid was needed and a dual luciferase assay, measuring both renilla and firefly luciferase was run for these plasmids. In paper I and II, no normalisation was used for transfection efficiency, however, the transfections were repeated several times with similar results.

### 3.1.9 RNA isolation, reverse transcription and quantitative real-time PCR (paper I-IV)

Total RNA isolation was performed using the RNeasy system (Qiagen) and reverse transcription was performed on 350-1000 ng of total RNA using a poly-dT primer and Superscript II or III (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using gene-specific TaqMan assays (paper I, II, IV) or PPARD exon-specific primers and probes (paper III). The reaction conditions involved an initial denaturation step for 10 min at 95°C, followed by 45 cycles of amplification with 15 sec at 95°C and 1 min at 60°C, using an ABI prism 7000 (Applied Biosystems). All samples were analysed at least in duplicates and the data was analysed using the comparative Ct-method.

For miRNA detection, the miRNeasy kit (Qiagen) was used for isolation of total RNA. Reverse transcription was performed on 10 ng total RNA using specific primers for miR-9, miR-29 or U48 together with the TaqMan miRNA reverse transcription kit (Applied Biosystems). The cDNA was amplified by real-time PCR with the corresponding TaqMan miRNA assays (Applied Biosystems) according to the instructions, using the conditions described above.

### 3.1.10 Coupled transcription/translation (paper I-III)

Coupled *in vitro* transcription and translation was performed to determine relative protein expression from different mRNA species (paper III) and to obtain the proteins used in the EMSAs (papers I-III). The TNT Quick coupled Transcription/Translation system (Promega) was used together with 1 μg of the TNT plasmid and either [$^{35}$S]methionine (Amersham) or unlabelled methionine according to the manufacturer's

instructions. The translation products were subsequently separated by a 10 % SDS-PAGE, subjected to autoradiography and analysed on a PhospoImager (paper II) or used in the EMSAs as described below.

### 3.1.11 Western blot (paper II-IV)

Whole cell extracts from primary hepatocytes treated with fenofibric acid (paper II) where subjected to Western blot analysis under reducing conditions as described by Laemmli [181], using an ALT1 specific antibody (paper II). Likewise, nuclear extract from HeLa cells transfected with PPARD1, PPARD2 or empty expression vector (paper III), or from monocytes treated with LPS or vehicle (paper IV), were subjected to Western blot analysis using a PPARD specific antibody, detecting the N-terminal part of the protein (sc-7197, Santa Cruz). Furthermore, *in vitro* translated PPARD from the different 5'-variants in paper III were subjected to Western blot analysis using an antibody detecting the C-terminal of PPARD (IMG 3297, Nordic Biosite). In paper IV, nuclear extracts from primary monocytes treated with LPS or vehicle for 24 hours were subjected to Western blot analysis, using precasted gradient gels 4-12 % (Criterion XT Bis-Tris Gels, BioRad) and the PPARD antibody sc-7197 (Santa Cruz).

### 3.1.12 Electrophoretic mobility shift (EMSA) (paper I-III)

EMSA was performed to study binding of the PPARs to putative PPREs (paper I and II) and also to study the binding of PPARD1 and PPARD2 to a classical PPRE (paper III). Double-stranded oligonucleotides corresponding to the putative PPREs, or to the classical rat acyl-CoA (ACO), were labelled with [$\gamma$-$^{32}$P] and mixed with a buffer containing 2 μg poly(dI-dC), 0.75 mM EDTA pH 8.0, 18 mM HEPES pH 7.9, 0.5 mM dithiothreitol and 4 % Ficoll and *in vitro* produced PPAR and RXR in a molar ratio of 3:1. For competition, 100-fold molar excess of unlabelled double-stranded oligonucleotide was added and for the supershift, the PPARD antibody sc-7197 was used. The mixtures were subjected to a 6 % polyacrylamide gel which was run 4h at 200 V, dried and then analysed on a PhosphoImager.

### 3.1.13 Chromatin immunoprecipitation (ChIP) (paper I-II)

ChIP was performed to assess *in vivo* binding of PPARD to the promoters of apoA-II and ALT1 (paper I and II). HepG2 cells were treated with PPAR agonists for 8 hours and the DNA and protein were subsequently cross-linked using formaldehyde. The chromatin was purified, followed by enzymatic digestion and immunoprecipitation

using a PPARA, G and D antibody, respectively, or a control IgG. Immunoprecipitated DNA was subjected to PCR with primer pairs specific to the putative PPREs in apoA-II and ALT1, respectively, whereas primer pairs amplifying regions further downstream were used as negative controls. Chromatin input was used as a positive control while $H_20$ was used as blank control templates for both primer pairs.

### 3.1.14 Rapid amplification of cDNA ends (RACE) (paper II)

Both 5'- and 3'-RACE were performed to identify possible alternative 5'-ends in transcripts of PPARD and to study alternative 3'-splicing. Marathon cDNA from placenta, adipose tissue and pancreas (Clontech) were used together with the adapter primers AP1 and AP2, together with combinations of PPARD-specific primers. Two sequential rounds of either 5'- or 3'-RACE were carried out and subsequently the obtained PCR products were purified and sequenced. Additional rounds of nested PCR were performed with the AP2 primer and exon-specific PPARD primers in order to enrich for transcripts expressed at low levels.

### 3.1.15 Human subjects (paper II)

The clinical study was a phase 1 study with the intentions to assess safety, tolerability, effects on lipids and pharmacokinetics of repeated oral doses of the PPARA agonist AZD4619. The study included 20 healthy males randomized to receive once-daily doses of 5 mg AZD4619 (n=15) or placebo (n=5) for 21 days. The ages of the participants were ranged from 20-29 years and their BMI were between 20 to 27.

### 3.1.16 Statistical Analysis (paper I-IV)

In order to establish differences in reporter vector activity or miRNA/mRNA expression between control and treated cells, Student's t-tests (two-tailed) were used (papers I-IV). For differences in the variables measured over time in the clinical data in paper II, the paired t-test was used. Significant differences are indicated as: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

# 4 RESULTS AND DISCUSSION

### 4.1 PPARD activation increases expression of the human apolipoprotein gene (paper I)

ApoA-II is one of the major apolipoproteins of the HDL particle but its anti-atherogenic role is not as established as for apoA-I [10]. However, recent data show that apoA-II is associated with a decreased risk of CVD [182]. The PPARA activating fibrates are shown to increase the levels of both apoA-I and apoA-II in plasma in humans, as well as the HDL cholesterol, supporting the role of apoA-II as anti-atherogenic. Moreover, obese rhesus monkeys treated with the PPARD agonist GW501516 show elevated plasma levels of apoA-I and apoA-II, as well as increased HDL cholesterol levels, further suggesting a beneficial role of apoA-II [95]. It is established that the effect of the fibrates on the apoA-II gene is mediated through a PPRE in the proximal promoter located at -737/-717, denoted the J-site [84]. In contrast, there is no information about the effects of PPARD activation on the apoA-II gene, hence we decided to investigate this matter. Treatment of HepG2 cells with the PPARD agonist GW501516 increased expression of apoA-II mRNA by 1.22-fold as measured by quantitative real-time PCR. Transient transfections of a 3 kb promoter construct of apoA-II in both HepG2 and HuH-7 cells showed that the effect was due to increased transcription and that a cotransfected PPARD plasmid potentiated this effect. The apoA-II promoter was analysed *in silico* to identify putative PPREs and, additional to the J-site, one putative PPRE was found at -2656/-2636, denoted the O-site. This site contained an equal number of identical nucleotides of the core PPRE consensus as the J-site. Furthermore, the O-site contained 4/7 matches in the 5'-flanking region compared to only 2/7 in the J-site (Fig. 11). Interestingly, sequential mutagenesis of these two PPREs in the promoter revealed that the effect of the PPARD agonist was mediated only through the J-site and not the O-site.


**Figure 11. Comparison of the putative PPREs in the apoA-II promoter**
Comparison of identified PPREs in the apoA-II promoter designated as the O-site and J-site, respectively, and the consensus PPRE sequence. The number of matches for the 5'- flanking region and the core DR1, respectively, compared to the consensus sequence are shown to the right. Identical nucleotides between the consensus sequence and the PPRE are denoted by an asterisk.

As previously reported [84] not only PPAR-induced activity was abolished when the J-site was mutated but also the basal activity decreased, reflecting the fact that the J-site is an important regulatory region also for other members of the nuclear receptor superfamily [183]. Both electromobility shift assays (EMSA) and chromatin immunoprecipitation assays (ChIP) confirmed the binding of PPARD to the J-site, establishing the role of apoA-II as a PPARD target gene. Increased transcription of the apoA-II gene is relevant for the abundance of apoA-II in plasma, since the apoA-II levels are controlled mainly by its synthesis rather than catabolism [184]. Whether the effects of an increase in apoA-II levels after PPAR agonist treatment adds on to the effects of the concurrent increase in apoA-I remains to be elucidated. The average apoA-I/apoA-II molecular ratio in human plasma is 2:1, which suggest that such an abundant protein should have important physiological functions [185]. Since increased synthesis of apoA-II is correlated with elevated plasma HDL concentrations, apoA-II might be one of many genes responsible for the beneficial effects regarding lipid and lipoprotein metabolism observed as a result of PPARD activation.

### 4.2 PPAR agonists regulate the ALT1 gene expression in human hepatocytes (paper II)

Even though ALT activity is used as a standard marker for hepatotoxicity in human clinical trials of pharmaceutical drugs, not much is known about the regulation of the two ALT genes, GPT1 and GPT2. The genes give rise to ALT1 and ALT2, respectively, which have similar enzymatic activities. ALT1 is highly expressed in liver, skeletal muscle and kidney, whereas ALT2 is found in skeletal muscle and heart but not in the liver and kidney [44]. Hence, ALT1 is the dominant isoform expressed in

the liver and it has been shown to be responsible for the basal ALT activity of human normal serum [44]. In a phase I clinical trial of AZD4619, a new PPARA activating compound developed by Astra Zeneca, the serum ALT levels increased over time in some of the participants. The trial went on for 21 days and at the last day of dose, five of the 15 participants had ALT levels above upper level of normal (ULN), defined as the 95 percentile of ALT activities from pre-dose (Fig. 12). At follow-up at day 31, seven of the participants were above ULN. Similarly, AST-levels increased in some of the participants during the study but no other liver markers, such as γ-glutamyl transferase, t- bilirubin, alkaline phosphatase, pro-thrombin or creatine kinase, were elevated. However, due to the increased ALT and AST levels, AZD4619 was discontinued from development.

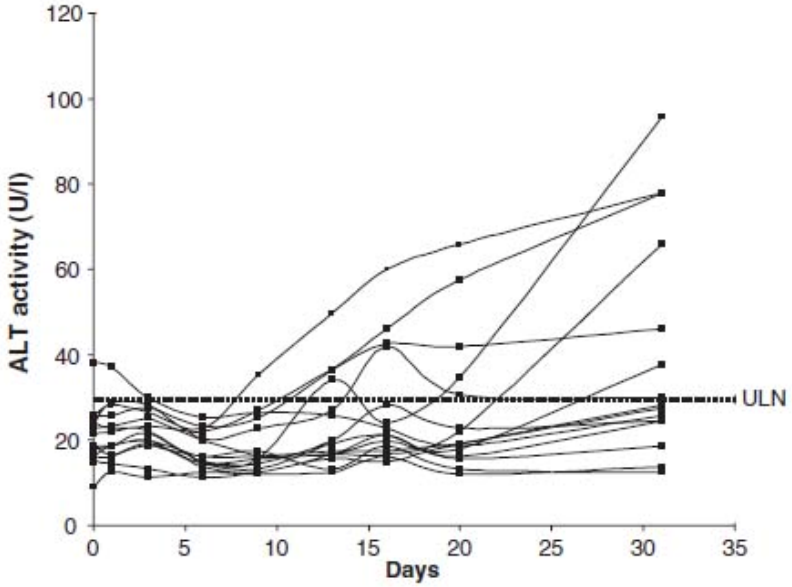


**Figure 12. Serum ALT activities in healthy volunteers treated with AZD4619**
15 human subjects were treated daily with 5 mg AZD4619 for 21 days. Serum ALT activities (U/l) increased after treatment with AZD4619 in some of the 15 subjects. ULN = upper level of normal

Since previous *in vitro* studies have shown that the AST gene is transcriptionally regulated by PPARA [186] we focused on testing the hypothesis that PPAR agonists regulate ALT gene expression. The PPARA agonist fenofibric acid was used as a model compound for PPARA activation and increased ALT1 expression in human primary hepatocytes compared to untreated cells, as measured by Western blot. Fenofibric acid treatment also increased mRNA expresson of ALT1 in three out of five different donors of primary hepatocytes measured by quantitative real-time PCR, indicating that the effect varies between individuals. Treatment of the hepatoma cell

line HuH-7 with fenofibric acid showed that ALT1 but not ALT2 mRNA expression was induced after the treatment compared to untreated cells. To establish if the effect was transcriptional, two kb of the GPT1 and GPT2 promoters, respectively, were cloned into a reporter vector and subsequently used in transient transfection assays in HuH-7 cells. Both fenofibric acid and AZD4619 showed dose-dependent increases of ALT1 promoter activity, whereas the ALT2 promoter did not respond. Thus, the effects of PPARA on ALT enzyme activity are accounted for by ALT1. Furthermore, PPARG and PPARD agonists were also shown to activate the ALT1 promoter, indicating that all the PPARs had similar effect on the promoter. In contrast, other potent compounds such as 12-O-Tetradecanoylphorbol 13-acetate (TPA) and TNFα did not activate the ALT promoter (Fig 13).

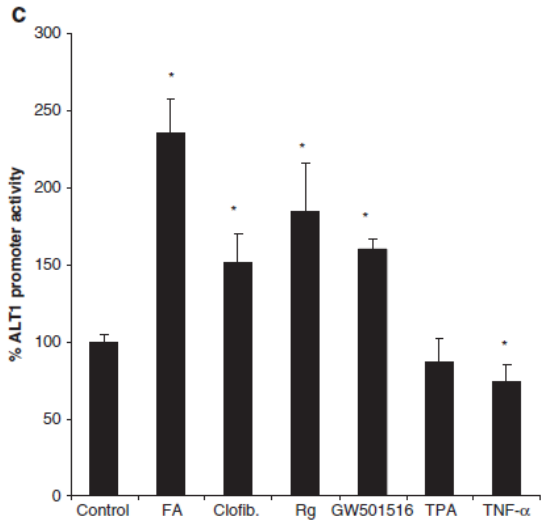


**Figure 13. Induction of ALT1 promoter by PPAR agonists in transfection assays**
PPARA agonists: Fenofibric acid (FA), Clofibrate (clofib.); PPARG agonist: Rosiglitazone (Rg); PPARD agonist: GW501516; Miscellaneous: 12-O-Tetradecanoylphorbol 13-acetate (TPA), tumour necrosis factor alpha (TNF-α).

Using *in silico* tools, the promoter of GPT1 was screened for PPREs and seven putative sites were found. EMSA studies verified that PPARG could bind to a site located at -574 in the promoter, whereas it could not bind to the other putative PPREs. Furthermore, mutation of the -574 site abolished the binding of PPARG in the EMSA and reduced the responsiveness to PPAR agonists of the promoter construct. Noticeably, PPARA did not bind to this site using EMSA, even though it could bind to the control rat ACO-PPRE. Importantly, PPARG is known to bind more strongly than the other PPARs to PPREs and the lack of binding of PPARA in this study might be due to suboptimal conditions of the EMSA or to the fact that the PPRE was weak [76].

Since EMSA only reflects binding to naked DNA, a ChIP was performed to evaluate the effects of the PPAR agonists in the presence of chromatin. The ChIP confirmed binding of both PPARA and PPARG to the -574 PPRE. This study shows that PPAR agonists can induce ALT1 by increased transcription of the ALT1 gene which is an alternative explanation for increased ALT protein in plasma.

Since ALT was introduced in clinical monitoring during the 1950s [43], much attention has been given to its role as a hepatotoxicity marker. In contrast, the function of ALT as a metabolic enzyme important for gluconeogenesis has not been given much notice. It is known that plasma ALT and AST levels increase in people experiencing a rapid weight loss, peaking after 2 weeks [187]. The extreme gluconeogenic condition is a possible explanation for the induction of the aminotransferases in the liver during fasting [188, 189]. Elevations in aminotransferase levels are also associated with obesity and the metabolic syndrome [190, 191], even though it is unknown whether it is the obesity per se which affect ALT activity or not. Furthermore, increased plasma ALT levels are shown to predict the incidence of type-2 diabetes and cardiovascular disease [191, 192]. Gluconeogenesis is increased in subjects with type-2 diabetes and the elevated ALT levels might be due to a an enzyme induction as the need for aminotransferases in the liver increases [193]. Thus, ALT elevations in plasma might not only reflect a hepatic injury but the metabolic status of the liver might also play a role for plasma ALT levels. In further support of this alternative hypothesis for ALT elevations is the fact that PPARA is important for gluconeogenesis. PPARA knock-out mice show impaired gluconeogenesis as a response to fasting compared to PPARA wild-type mice [82]. Hence, it is likely that activation of PPARA agonists would induce ALT expression in the liver. Furthermore, moderate but transient elevations in ALT and AST levels are seen in approximately 10 % of the patients treated with fenofibric acid without any other signs of liver injury [50, 51]. This individual variation was also observed in our study where only some individuals responding to AZD4619 treatment with increased aminotransferase levels. Also, there was a difference in the response in ALT expression after treatment with fenofibric acid between the different hepatocyte donors. The PPARG agonists have never been associated with transaminase elevations in the clinic, unless they have been proven to be toxic, as in the case with Troglitazone. Instead PPARG agonists are shown to reduce ALT activity in serum [194], probably due to the fact that they improve the insulin sensitivity, which reduces gluconeogenesis in the liver [195]. The alternative hypothesis of ALT induction by drugs influencing

metabolism was recently demonstrated to be valid also *in vivo* [196]. Rats treated with dexamethasone for 24 hours showed increased levels of ALT activity in the serum and in the livers compared to untreated rats, without any signs of tissue damage within this time. The dexamethasone treated rats also showed increased amounts of glycogen in their livers, indicating an increased gluconeogenesis, which reflects the condition induced by the treatment.

Of note, the moderate and transient increase in ALT activity induced by the PPAR agonists should be distinguished from the massive increases that occur during liver damage. The development of additional hepatotoxicity markers would be important for use in combination with ALT so that the new PPAR compounds have a chance to pass the clinical monitoring of today.

### 4.3 Regulation of PPARD by alternative splicing (paper III)

Since the majority of studies on PPARD concern the effects of its activation, we decided to investigate its regulation and more specifically; whether it is subjected to alternative splicing, which might give rise to additional isoforms and/or influence the expression. The human PPARD gene consists of 9 exons of which exons 1-3, the 5'-end of exon 4 and the 3'-end of exon 9 are untranslated [92]. In this study, five new alternative exons were identified in the PPARD gene by 5'-RACE using cDNAs from placenta, adipose tissue and pancreas and these exons make up a variable range of PPARD mRNA species (Fig.14 ). TaqMan analysis using a panel of human cell lines and tissues revealed that the most common splice variant encompassed exon 2 connected to exon 4 (Fig 14, B), whereas the other splice variants were expressed at much lower levels. *In vitro* transcription/translation and Western blot analysis showed that the various transcripts were translated into PPARD protein with different efficiencies, inversely correlated to the length, as well as the number of AUGs in the 5'-UTR. Analysis of basal activity of the previously identified promoter upstream of exon 1 and the alternative promoters upstream of the newly identified putative transcription start sites (Fig 14, H-K) in transient transfection assays, revealed that the promoter upstream of exon 1 is the major region for transcriptional activation.

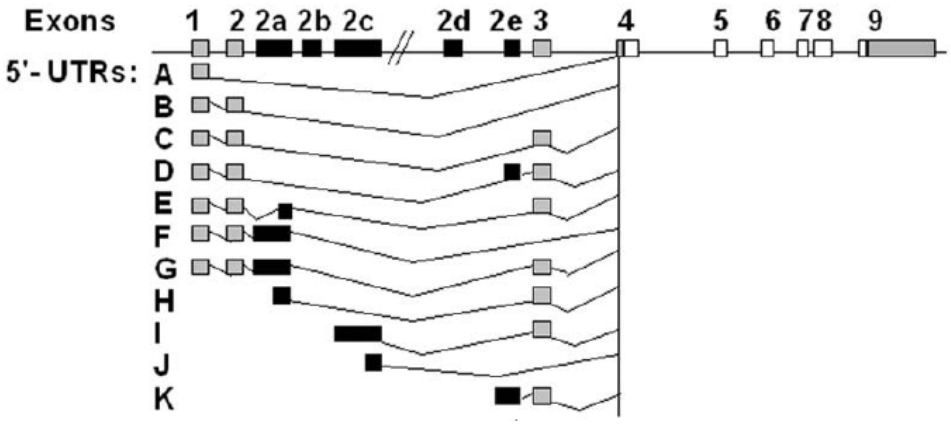


**Figure 14. A schematic representation of the PPARD gene**
Coding exons (*white* boxes), previously reported untranslated exons or part of exons (*grey* boxes), and herein identified untranslated exons (*black* boxes). The variety of splicing among untranslated exons and alternative 5'-ends identified by 5' RACE is shown below the gene (*5'-UTRs: A-K*).

Moreover, a 3' splice transcript encoding a truncated isoform of PPARD, PPARD2, was identified in placenta and adipose tissue. This truncated variant is formed due to intron retention of intron 8 which introduces an early stop codon, leading to a truncated PPARD protein lacking 82 amino acids of the C-terminal containing the ligand-binding domain. Analysis of PPARD2 in transient transfection assays revealed that it could not be transactivated by ligand-binding but rather repressed ligand-induced activation of PPARD1, constituting a dominant negative inhibitor of the full-length receptor. Furthermore, PPARD2 was unable to bind to a classical PPRE in EMSA studies, suggesting that it does not compete with PPARD1 for DNA-binding as is suggested for the dominant negative full-length mouse orthologous of PPARD (E411P) [197, 198]. The mechanism of inhibition of PPARD2 might instead be due to competition for cofactors or its heterodimerization partner RXR, which has been speculated as the negative regulatory mechanism for some of the other truncated nuclear receptors [132, 133, 135, 136].

The diversity in PPARD 5'- transcripts described in this study adds another level of regulation. Post-transcriptional regulation by 5'-splicing have already been described for the mouse PPARD [199] and also for the human PPARA and PPARG genes [200, 201] where the PPARG transcripts also have shown to influence translational efficiency [202]. The mechanism which accounts for differences in translational efficiency between the 5'-splice variants of PPARD is not clear but the fact that long 5'-UTRs and number of AUGs play a role indicate that secondary structures of the 5'-UTR or upstream open reading frames (uORF) might be involved [203]. A study regarding

alternative 5'-transcripts of PPARG identified that the stability of the 5'-UTRs is inversely related to the translational efficiency [202]. Even though alternative promoters have been described to be functional in the mouse PPARD gene [199], the promoter analysis in this study showed low activity compared to the one already described. However, it is possible that the promoters described here might be functional in tissues other than the ones tested in this study, due to differential expression of cofactors. Truncated isoforms have been described for the other members of the PPAR family and for many of the other nuclear receptors. The mechanism of inhibition of PPARD2 remains to be elucidated but general mechanisms suggested for the other nuclear receptors are; competition for binding to the DNA, competition for cofactors/auxiliary factors or for heterodimerization partners, or influences on nuclear localisation [132-136]. The conclusion of this study is that alternative splicing of human PPARD could constitute an intrinsic role for the regulation of PPARD expression and activity, which could be of relevance both in physiology and disease.

### 4.4 Regulation of PPARD by miRNA (paper IV)

In order to further investigate the mechanisms governing the regulation of the human PPARD gene, the 3'-UTR of PPARD was analysed *in silico* for miRNA binding sites using the UCSC genome browser. Two putative miRNA target sites were identified, miR-9 and miR-29 (Fig 15).

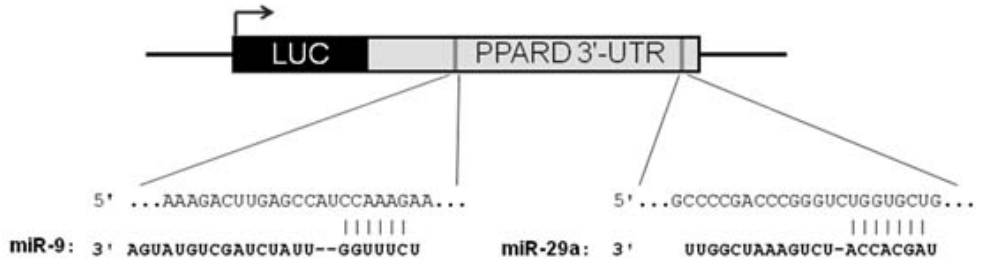


**Figure 15. Schematic representation of the PPARD 3'-UTR luciferase reporter construct**
Putative target sites for miRNAs and their corresponding miRNA sequences are shown below.

Transient transfection assays in HEK293 cells using a PPARD 3'-UTR reporter construct showed decreased luciferase expression when miR-9 was cotransfected, whereas miR-29 or the control miR-155 did not alter the luciferase activity, indicating that miR-9 can regulate the PPARD gene. Furthermore, when the miR-9 target site was mutated, the effect of miR-9 was abolished, demonstrating that the regulation of the 3'-UTR by miR-9 was mediated through the *in silico* identified miR-9 site (Fig 16).

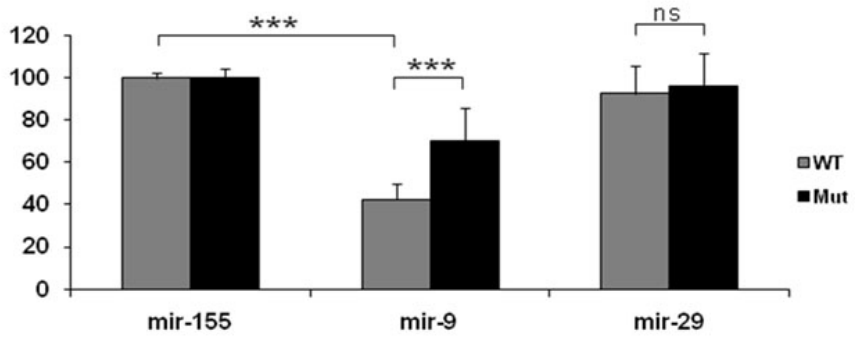


**Figure 16. The PPARD gene is a direct target of miR-9**
Luciferase activity of reporter constructs that contain the wild-type (grey bars) of the entire PPARD 3'-UTR or mutated at the putative miR-9 binding site (black bars). HEK293 cells were transiently cotransfected with the PPARD 3'-UTR reporter constructs and expression vectors coding for miR-155, miR-9 or miR-29 as indicated.

Moreover, a luciferase reporter containing only the short sequence coding for the miR-9 target site (36 bp) also showed decreased luciferase activity after coexpression of miR-9, whereas mutation of the seed sequence abolished the luciferase activity. Also, cotransfection of an antisense of miR-9, antagomiR-9, in the absence of any cotransfected miR-9, showed an increase in luciferase activity in cells transfected with the PPARD 3'-UTR or the short miR-9 construct. Furthermore, when miR-9 was transfected into the cells, the luciferase activity could be restored to almost normal levels by antagomiR-9. These results strongly support the finding that miR-9 represses PPARD protein expression. In contrast, miR-29 did not affect the expression of the full-length 3'-UTR PPARD reporter, indicating that it is not a regulator of PPARD. To elucidate whether miR-9 targets only the PPARD protein levels or if mRNA levels also are affected, miR-9, miR-29 or the empty plasmid was transfected into HEK293 cells for 24 h and PPARD mRNA expression was measured by quantitative real-time PCR. However, no difference between the groups could be detected which suggests that miR-9 does not affect the levels of the PPARD transcript, instead it inhibits the translation of the mRNA into protein.

Since miR-9 has been shown to play an important role in the inflammatory response in monocytes [204], the relevance of miR-9 regulation in relation to PPARD expression was investigated in monocytes and macrophages. Based on the knowledge that treatment of primary monocytes with LPS increases miR-9 expression [204], human primary monocytes were treated with LPS to study the effects on PPARD function. Accordingly miR-9 expression as well as PPARD mRNA expression was increased

after both 8 and 24 hours treatment. However, the PPARD target genes adipose differentiation-related protein (ADFP), adipose triglyceride lipase (ATGL) and carnitine palmitoyltransferase (CPT1) were notably lower after 8 hours treatment and only increased after 24 hours, suggesting that the increased expression of miR-9 in the cells was counter-acting the effects of the PPARD elevation. Western blot analysis of monocytes treated with LPS for 24 hours did not show any major differences after LPS treatment compared with cells only treated with vehicle, further proposing that PPARD protein levels were not as increased by the LPS treatment as the PPARD mRNA expression.

Due to the recent identification of PPARD as a major regulator of the M2 phenotype of macrophages, the involvement of miR-9 in the alternative activation of macrophages was elucidated. Human primary monocytes were differentiated into macrophages and subsequently treated with LPS and IFNγ to become M1 macrophages, IL-4 to become M2 macrophages or vehicle to serve as control (M0) for 6, 24 or 72 h. Of note, PPARD mRNA expression was higher in M1 compared to M2 macrophages at 24 and 72 hours, whereas mRNA expression of PPARG was higher in M2 compared to M1 at all time points measured. Analysis of miR-9 expression revealed no statistical difference between the treatments suggesting that miR-9 expression is not involved in the development of different macrophage phenotypes.

MiR-9 is a microRNA which has been reported to be involved in distinct processes such as neuronal differentiation in the brain, release of insulin from the pancreas and the immune response in monocytes [204-206]. By increasing concomitantly with NFκB in monocytes and targeting its 3'-UTR, miR-9 inhibits the inflammatory cascade and constitutes a negative feed-back loop of the inflammatory response [204]. Similarly, this study shows that PPARD gene expression is increased in monocytes during inflammatory stimulation with LPS and the protein expression is partly inhibited by the simultaneous increase in miR-9. The importance of PPARD for the inflammatory status of macrophages is not clear but recent studies in murine macrophages suggest a function of PPARD in inflammation, by causing a switch from M1 to M2 phenotype [29, 30]. However, in this study PPARD expression was higher in M1 than M2 macrophages, which supports data from a recent human study [31], highlighting the differences between human and mouse macrophages. The lack of differences in

miRNA expression between the macrophage subtypes suggests that the function of miR-9 is of importance only in monocytes but not in differentiated macrophages.

# 5 GENERAL DISCUSSION

PPARD is a protein with multiple functions in basically all the tissues of the human body. When the PPAR family was discovered in the early 1990:ies, PPARD was not considered to be more than a house-keeping gene in the shade of PPARA and PPARG, the important mediators of the fibrates and glitazones, respectively. Today, when detrimental side-effects associated with some of the PPARG agonists have been shown and the role of PPARD as a nuclear receptor with beneficial effects on both lipid metabolism and insulin sensitivity have become more clear, a number of studies regarding its functions have emerged. The increased oxidative capacity in muscle, increased fatty acid combustion in adipose tissue and beneficial effects on lipoprotein metabolism are clear indicators of overall positive effects mediated by PPARD, which could be taken advantage of in the treatment of obesity and type-2 diabetes. However, caution has to be taken since PPARD has been reported to increase cell proliferation and to be involved in cancer formation in the colon, even though the literature on the subject is not totally conclusive [207] .

The first two studies of this thesis further supports the role of PPARD as an important regulator of human lipoprotein composition and overall metabolism. The increased expression of apoA-II mediated by transactivation of PPARD is likely one of the reasons for the increase in the number of HDL particles and the enhanced reverse cholesterol transport in primates and humans treated with GW501516. The activation of the ALT1 gene in the liver by all three PPARs implies that increases in lipid oxidation results in a state of the body that favors gluconeogenesis including the enzymes implicated in that process. Hence, PPAR activation mimics a state of starvation of the body which could be taken advantage of in obesity and obesity-related disorders such as type-2 diabetes and cardiovascular disease. However, if the ALT enzymes are to be assessed during clinical trials of developing PPAR agonists, an awareness of the properties of the PPARs to induce aminotransferases by the non-toxic mechanism is important.

The effects of PPARD have mainly been investigated using activation of the receptor by a specific ligand, or by studying the effects of PPARD knock-out or transgenic mice models. These studies show the importance of PPARD activation in lipid metabolism

but have also identified an inflammatory role of the receptor in macrophages. As demonstrated by the LDL receptor knock-out mouse model, a reduction in plaque size is seen both after knock-out of myeloid PPARD and after PPARD activation, due to its sequestering of inflammatory corepressors in the unliganded state [73]. Hence, the effects of regulation of the gene are complex, since not only abundance of the protein but also availability of ligands is important for its downstream effects. Additionally, the availability of coactivators and corepressors, as well as availability of the other PPAR family members or other nuclear receptors might determine the response of PPARD expression or activation [71, 72]. Hence, studies of this nuclear receptor have to be performed in a well controlled experimental setting. No detailed investigations on the regulation of the human PPARD gene has been performed until now, even though the mouse gene has been reported to be regulated by alternative promoter usage and alternative splicing [199]. In this thesis, human PPARD is shown to be subjected to alternative splicing in the 5'-end, which results in differences in translational efficiency between the different transcripts. Furthermore, a 3'-truncated PPARD isoform, PPARD2, is shown to be a negative regulator of the full length variant. These findings could be of importance for PPARD expression in different tissues or during different metabolic conditions, however, the functional relevance of the splice variants remains to be identified. Furthermore, the mechanism of negative regulation of PPARD2 still remains to be elucidated.

Regulation of PPARD by miRNA was also identified as a posttranscriptional regulatory mechanism of the human PPARD gene. However, to understand the importance of PPARD regulation by miR-9, further studies of PPARD function in monocytes is needed. Since miR-9 is highly expressed in the brain and PPARD expression is important for myelination of the corpus callosum [100], the brain might be one of the main sites of PPARD regulation by miR-9. Additionally, the concomitant increase of PPARD and miR-9 in monocytes after inflammatory stimulation suggests a tight relationship between these two players during the initial inflammatory response of monocytes, and might be relevant in the initiation of the atherosclerotic process.

The future for PPARD as a drug target is not easy to predict. With the declining interest for the PPARA and PPARG agonists on the market, a PPARD compound might take over as their replacement therapy. However, the aminotransferase elevations of PPARA agonists in clinical trials as well as the failures of some of the PPARG agonists which

have been on the market for many years, the PPARs in general have a bad reputation. Also, non-resolved issues about its involvement in tumour formation are of major concern, even though there has been no indication of increased tumourogenesis in the millions of patients taking fibrates or glitazones. Another obstacle for the development of PPAR agonists is the fact that all new PPAR ligands require two years of preclinical oncogenicity studies prior to initiating any clinical studies of 6 months and longer, which might not make the effort worth-while for pharmaceutical companies [118]. However, tissue-specific PPAR modulators (SPPARMS) might be the resolution to this problem. The future might hold a muscle-specific PPARD modulator that will be prescribed additionally to exercise to patients with the metabolic syndrome. The exercise itself would increase PPARD expression in the skeletal muscle and the drug would cause its activation, resulting in increased expression of genes involved in lipid oxidation. The increased oxidation of fatty acids in the muscle increases the overall insulin sensitivity, and is beneficial for the endurance capacity of the muscle fibers. Of note, not the PPARD agonist itself but the combination of exercise and PPARD agonist is important for the beneficial effects, as recently demonstrated by a study of sedentary mice administrated either with GW501516 alone or in combination with an exercise program [208]. Exercise not only increases PPARD expression in skeletal muscle but also improves the metabolic status of the patients, which might be relevant for the synergic function of PPARD activation and exercise. An alternative might be to influence the splicing machinery to only create short 5'-ends of PPARD mRNA, hence increasing the translational efficiency. Whether that is applicable or not is too early to say but the idea is intriguing. Treatment with an antagomiR-9 might also be a way to increase PPARD expression. However, miR-9 have additional targets, both known and unknown which probably would cause numerous side-effects. To be able to modulate this receptor in a clinical setting, it is important to further elucidate by which mechanisms it is regulated. With increasing knowledge about different functions of PPARD, additional studies on the regulation of this nuclear receptor will probably emerge to resolve new treatments for metabolic syndrome and CVD. Until then, exercise in combination with a high dietary intake of PUFAs will be the most effective way to increase the expression of PPARD and subsequently cause its activation, not only to treat but also to prevent the development of the metabolic syndrome.

# 6 CONCLUSIONS

The work of this thesis has identified new target genes of PPARD and also revealed post-transcriptional mechanisms of regulation of this nuclear receptor. In summary, the conclusions of this thesis are;

- **Paper I:** PPARD activation increases the expression of the human apoA-II gene, which might be one of the reasons why treatment with PPARD agonist increases production of HDL particles and stimulates reverse cholesterol transport.
- **Paper II:** The ALT1 gene is a transcriptional target of the PPARs, which may result in mild ALT elevations in plasma in the absence of liver injury when assessing new PPAR agonists in clinical trials.
- **Paper III:** PPARD is subjected to alternative splicing, both in the 5'- and 3'-ends, which identifies possible mechanisms for PPARD regulation in time and space.
- **Paper IV:** The human PPARD gene is regulated by miRNA-9, which might be important for the early inflammatory response in monocytes.

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"What fits your busy schedule better, exercising one hour a day or being dead 24 hours a day?"

(Adapted from www.hapi-naples.com)

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